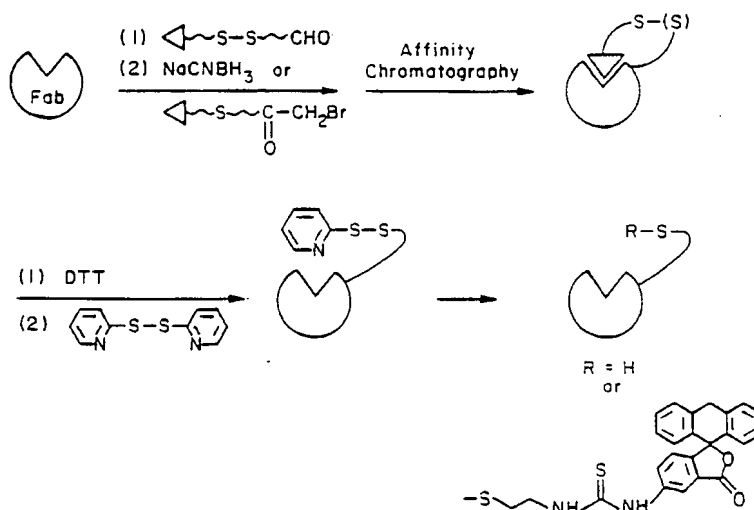




INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : C07K 17/06, C12N 9/96	A1	(11) International Publication Number: WO 90/05749 (43) International Publication Date: 31 May 1990 (31.05.90)
(21) International Application Number: PCT/US89/05257 (22) International Filing Date: 15 November 1989 (15.11.89) (30) Priority data: 273,786 18 November 1988 (18.11.88) US (71) Applicant: THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 300 Lakeside Drive, 22nd Floor, Oakland, CA 94612 (US). (72) Inventor: SCHULTZ, Peter ; 6331 Valley View, Oakland, CA 94611 (US). (74) Agent: HESLIN, James, M.; Townsend and Townsend, One Market Plaza, 2000 Steuart Tower, San Francisco, CA 94105 (US).		(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK, FI, FR (European patent), GB (European patent), IT (European patent), JP, KR, LU (European patent), NL (European patent), SE (European patent), SU. Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>

(54) Title: CONJUGATED POLYPEPTIDES AND METHODS FOR THEIR PREPARATION AND USE



(57) Abstract

Polypeptide compositions are provided having a binding site specific for a particular target ligand and further having an active functionality proximate the binding site. The active functionality may be a reporter molecule, in which case the polypeptide compositions are useful in performing assays for the target ligand. Alternatively, the active functionality may be a chemotherapeutic agent, in which case the polypeptide compositions are useful for therapeutic treatment of various diseased states. A novel method for preparing such polypeptides having active functionalities proximate their binding site comprises combining the polypeptide specific for the target ligand with an affinity label including ligand having a reactive group attached thereto. The reactive group is then covalently attached to an amino acid side chain proximate the binding site and cleaved from the substrate. The substrate is eluted, leaving a moiety of the reactive group covalently attached to the polypeptide. The active functionality may then be attached to the moiety.

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CONJUGATED POLYPEPTIDES AND
METHODS FOR THEIR PREPARATION AND USE

5 This invention was made with Government support under Grant Contract No. AI-24695, awarded by the Department of Health and Human Services, and under Grant Contract No. N 00014-87-K-0256, awarded by the Office of Naval Research. The Government has certain rights in this invention.

10 Application serial number _____ (attorney docket no. 2307U-255), filed on the same date as the present application and naming Peter Schultz as the sole inventor, contains related subject matter. The entire disclosure of this related application is
15 incorporated herein by reference.

BACKGROUND OF THE INVENTION

1. Field of the Invention

20 The present invention relates generally to polypeptide compositions having active functionalities covalently attached proximate a binding site. More particularly, the invention relates to polypeptides wherein the active functionality is a reporter molecule or a chemotherapeutic agent, and to methods for the preparation of such polypeptides.

25 Numerous biological proteins are capable of specific attachment to other biological molecules as a result of intermolecular binding forces, including electrostatic bonds, hydrogen bonds, and van der Waals bonds. Such bonding occurs at a site on the protein
30 generally referred to as a binding site, and binding occurs only with target ligands having a specific molecular geometry which is complementary to the binding site. Examples of such interactions include antibody-antigen binding, enzyme-substrate binding,
35 hormone-receptor binding, lymphokine-lymphokine receptor binding, and the like. The availability of such specific binding interactions is relied on in a

variety of scientific, medical, and commercial processes, including immunoassays, therapeutic treatments, immunohistochemical methods, protein separations, reaction catalysis through transition state stabilization, and the like.

In many instances, it would be desirable to equip a specific binding protein or polypeptide with a constituent or functionality proximate the binding site, where the constituent will be able to provide a desired interaction with the target ligand. For example, it will often be desirable to provide constituents which are detectable or which have therapeutic or catalytic activity proximate a polypeptide binding site specific for a particular target ligand. By providing chemotherapeutic agents proximate the binding site (and absent from other locations) on specific binding proteins capable of binding diseased cells, chemotherapeutic activity may be enhanced while reducing toxic and other side effects which might otherwise be present. By providing a reporter molecule proximate a protein binding site (and absent from other locations) specific for a particular substance, assays may be provided where the presence of substrate provides for direct modulation of the observed signal from the reporter molecule.

Heretofore, methods for covalently binding active functionalities onto specific binding proteins, such as antibodies, have generally not been localized near the binding site. That is, binding has been achieved through amino acid side chains and carbohydrates which may be found at various locations on the protein surface, not limited to locations proximate the binding site. While enzymes have been modified to include functionalities, such as cofactors, proximate active sites, the localized binding of reporter molecules and chemotherapeutic agents proximate a protein binding site has not generally been

5 taught. Moreover, methods for localized binding of active functionalities to proteins which have not been structurally characterized are generally unknown, regardless of the type of protein and type of active functionality.

10 In view of the above, it would be desirable to provide polypeptides having binding sites wherein chemotherapeutic agents or reporter molecules are bound proximate the binding site and substantially absent from the remainder of the polypeptide. It would be further desirable to provide methods for covalently binding active functionalities, including reporter molecules, chemotherapeutic agents, catalytic groups, reactive groups, and the like, proximate the binding site on polypeptides, even when the structure of the polypeptide is unknown.

2. Description of the Relevant Art

15 Immunotoxins comprising toxins and drugs covalently bound to antibodies specific for cellular receptors and other targets are known. See, e.g., U.S. Patent Nos. 4,340,535; 4,368,149; 4,489,710; and 4,671,958. None of these patents, however, disclose site-specific binding of the toxins and drugs proximate the antibody binding site. European patent application 20 187 658 discloses antibodies covalently attached to activators. The antibodies bind to diseased sites within a host and the activators interact with an inactive substance within the host to produce an active substance. Binding of the activators is not limited to the antibody binding site. U.S. Patent No. 3,817,752, describes homogeneous enzyme immunoassays employing detectable enzymes having hapten bound thereto. By exposing the enzymes to antibodies specific for the hapten, modulation of the enzyme activity can be 30 achieved. The use of spin labels bound to enzymes to study enzyme activity is disclosed in Jones et al. (1973) Eur. J. Biochem. 34:28-40. Polyclonal

antibodies have been generated with cofactor binding sites (Raso and Stollar (1975) Biochemistry 14:584-591). The generation of monoclonal antibodies having a cofactor binding site is described in Shokat et al. (1988) Angew. Chem. 100:1227-1229. The generation of affinity labels for antibody combining sites is described by Kohen et al. (1980) FEBS Lett. 111:427-431; Metzger et al. (1970) Biochemistry 9:1267-1278; and Givol et al. (1971) Biochemistry 10:3461-3466. Site-directed mutagenesis has been used in conjunction with high resolution x-ray crystallography to analyze the structure of enzyme binding sites and the function of such sites in catalysis. Wilkinson et al. (1984) Nature 307:187-188; Craik et al. (1985) Science 228:291-297; Schultz et al. (1985) Biochemistry 24:6840-6848; Dalbadie-McFarland (1982) Proc. Natl. Acad. Sci. USA 79:6409-6413; and Sigal et al. (1984) J. Biol. Chem. 259:5327-5332. A cysteine in the active region of the enzyme papain has been modified with a flavin cofactor. Kaiser et al. (1984) Science 226:505-510. A thiol has been introduced into the enzymes staphylococcal nuclease and RNase S and subsequently derivatized with an oligonucleotide. Corey et al. (1987) Science 238:1401-1403. The active site serine of subtilisin has been chemically converted to a cysteine. Bender et al. (1966) J. Am. Chem. Soc. 88:3153-3154 and Koshland et al. (1966) Proc. Natl. Acad. Sci. USA 56:1606-1611.

SUMMARY OF THE INVENTION

According to the present invention, polypeptides are provided having active functionalities bound proximate a binding site, wherein the active functionalities are selected from the group consisting of reporter molecules and chemotherapeutic agents. The active functionalities are substantially absent from locations on the polypeptides other than proximate the binding site, and the presence of the functionalities

does not substantially interfere with ligand binding to the binding site. Polypeptides will usually be in the form of antibodies or antibody fragments, where the antibodies are capable of binding particular target ligands, but other binding protein forms may also find use, including enzymes, hormones, lymphokines, lectins, avidin, membrane-bound cellular receptors, soluble receptors, viral coat proteins, structural proteins, and the like. Polypeptides having a binding site specific for an analyte of interest may be joined to reporter molecules such as fluorophores, chemiluminescers, bioluminescers, dyes, spin labels, flavins, piezoelectric molecules, biotin, redox active molecules, and the like, and will be useful for performing assays where the activity or detectability of the reporter molecule is directly modulated by binding to the analyte. Polypeptides having binding sites specific for diseased cells, tumor antigens, pathogens, and the like, may be joined to chemotherapeutic agents, such as toxins, bactericides, radical scavengers, radical generators, oxidants, alkylating agents, and the like, and will be useful for therapeutic treatment where the activity of the chemotherapeutic agent may be maximized and side reactivity minimized.

In a second aspect of the present invention, methods are provided for preparing detectable and chemotherapeutic polypeptides by post-translational modification of the polypeptides to introduce the active functionality of interest. The polypeptides may be natural or synthetic, where synthetic polypeptides include those prepared by both recombinant and solid phase synthesis techniques. The synthetic polypeptides may be modifications of an exemplary natural polypeptide where one or more amino acids are substituted or added proximate the binding site. By introducing rare or non-natural amino acids proximate

the binding site and specifically attaching the active functionality through the rare or non-natural amino acid, non-localized binding of the functionality to sites in the polypeptide remote from the binding site may be minimized or avoided. The introduction of non-natural amino acids proximate the binding site is of particular interest as they can afford unique sites of reactivity which can assure that the active functionality being introduced will attach only at the location proximate the binding site.

When modifying natural polypeptides, i.e., those isolated from natural sources, the present invention provides a novel method which assures localized binding of the active functionality proximate the binding site. The method is useful even in the absence of structural information on the polypeptide. The method utilizes "affinity labels" which comprise a first reactive group cleavably bound to a ligand through a cleavable linker, where the ligand is specific for the binding site of the polypeptide. The first reactive group is capable of covalent binding to an amino acid side chain, usually a common side chain which will be expected to be present proximate the binding site (and elsewhere) on the polypeptide. By combining polypeptide with the affinity label, the ligand will form a complex with the binding site, thus carrying the first reactive group to a location proximate the binding site. The first reactive group may then be covalently attached to the amino acid side chain proximate the binding site. The reactive group is then cleaved from the polypeptide, and the ligand removed from the polypeptide. The polypeptide is left with a moiety (artifact) of the first reactive group covalently attached proximate the binding site. In some cases, the moiety itself may be the active functionality. More commonly, however, the moiety will serve as an attachment site for joining the active

functionality of interest. The active functionality will be joined to the moiety attachment site under conditions which minimize or avoid entirely attachment elsewhere on the polypeptide. This method for
5 attaching active functionality is not limited to reporter molecules and chemotherapeutic agents, and is useful for a wide variety of functionalities including catalytic functionalities and reactive functionalities, as described in copending application serial no.

10 _____ (attorney docket no. 2307U-255), filed on the same date as the present application, the disclosure of which is incorporated herein by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

15 Fig. 1 illustrates the cleavable affinity labels used in Example 1 of the Experimental section.

Fig. 2 outlines the methods for synthesizing the affinity labels of Fig. 1.

20 Fig. 3 illustrates the strategy for introducing a thiol functionality into the binding site of MOPC 315 in Example 1.

25 Fig. 4 is a high pressure liquid chromatography profile of the tryptic digest of the heavy chain of Fab labeled with 2 (Fig. 1) monitored by UV absorbents (solid line) and radioactivity (dashed line), as described in Example 1.

Fig. 5 illustrates the DNP-containing esters utilized as substrates for reaction with thiolated Fab in Example 1.

30 Fig. 6 is an Eadie-Hofstee plot of cleavage of ester 14 by thiolated Fab labeled with 2 in Example 1.

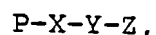
35 Fig. 7 illustrates the results of a fluorescence quenching binding assay for DNP-glycine with fluorescein-Fab adduct (\square) versus control with fluorescein plus underivatized Fab (Δ). Fluorescence quenching experiments were carried out at 10°C using 492 nm for excitation and measuring emission at 521 nm.

The fluorescein-Fab adduct was diluted with assay buffer (above) to 0.10 μ M. Aliquots of 2,4-DNP-glycine were added and, after mixing, the fluorescence observed. Free N-fluoresceinthioureido-2-mercapthoethylamine and underivatized Fab, each at 0.10 μ M, were treated in a similar experiment.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Novel polypeptides having binding sites capable of specifically binding a predetermined target ligand include at least one active functionality proximate the binding site. Locations on the polypeptide other than the binding site are substantially free from the active functionality so that the activity provided by the functionality will be localized to the binding site. The active functionality may be a reporter molecule, whereby the polypeptides will be useful in detecting the predetermined target ligand in a sample suspected of containing such ligand. Binding of the ligand to the polypeptide will modulate the activity provided by the active functionality, allowing detection and (optionally) quantitation of the substrate present in the sample. Alternatively, the active functionality may be a chemotherapeutic agent, whereby the polypeptide will be useful in treating a diseased state by site-specific drug delivery. Localization of the drug proximate the binding site will increase the efficiency of drug delivery by reducing the total drug dosage required. Side effects of the drug related to non-specific delivery will also be reduced.

The polypeptides of the present invention will have the following formula:



wherein P, X, Y, and Z are defined as follows.

P is a polypeptide having a binding site capable of specifically binding the target ligand of interest. The polypeptide may be natural or synthetic,

with the term natural generally referring to polypeptides isolated from living biological organisms, e.g., cell culture, animal tissue, plant sources, and the like, and the term synthetic generally referring to polypeptides produced by recombinant DNA methods and by chemical synthesis techniques, e.g., solid phase synthesis techniques. The polypeptides may be a single chain, or include multiple chains, and may be glycosylated or free from glycosylation. The size of the polypeptide is not critical, with polypeptides being in the range from 2 kilodaltons (kD) to 1000 kD, usually being in the range from 20kD to 200 kD. The polypeptides may be in the form of natural biological proteins, such as antibodies, enzymes, hormones, lectins, lymphokines, avidin (including streptavidin), membrane-bound cellular receptors, soluble receptors, viral coat proteins, structural proteins, and fragments thereof, as well as other proteins capable of specifically binding target molecules (ligands) which include analytes; biotin; diseased cells; tumor cells, normal cells, particularly classes of normal cells (e.g., T-lymphocytes); bacterial, fungal, and viral pathogens; parasites; mycoplasma; and the like. Of particular interest are antibodies which have been raised against the target ligand of interest.

Optionally, synthetic polypeptides may be prepared wherein the structure of a natural antibody (i.e., one which has been elicited against an immunogen) or other natural protein may be modified in a predetermined manner in order to facilitate introduction of the active functionality of interest, e.g., amino acids may be added, deleted, or substituted in order to provide a unique or rare attachment site proximate the binding site. Such modified polypeptides will generally retain the ability to mimic the naturally-occurring protein from which they have been derived and, in particular, will retain the ability to

bind to the target ligand of interest. In the case of active proteins, such as enzymes, hormones, lymphokines, and the like, it will frequently be desirable to reduce or eliminate their activity so that the corresponding polypeptide is able to bind the target of interest without performing the function normally associated with the protein.

Alternatively, the polypeptides of the present invention may assume novel configurations which are not characteristic of known classes of biological proteins. In such cases, the polypeptides will be synthesized, either by recombinant techniques or by solid phase synthesis techniques, to provide a product having a desired sequence and structural configuration which will allow for binding of the polypeptide to the target ligand of interest.

Regardless of form and source, it is necessary that the polypeptide have a binding site with affinity for the target ligand of interest. The affinity will be at least about 10^{-3} M^{-1} , usually being at least about 10^{-4} M^{-1} , preferably being at least about 10^{-5} M^{-1} , and more preferably being at least about 10^{-6} M^{-1} or higher. In the case of modified polypeptides, i.e., those which have changes in their amino acid sequence relative to a natural or exemplary protein, there should be no substantial loss of binding affinity as a result of the modification, and the modified polypeptide should generally meet the affinity levels just set forth even if some loss of affinity has occurred.

Polypeptides having the desired affinity are most easily prepared by raising antibodies, particularly monoclonal antibodies, against the target ligand or an analog thereof. The antibodies may be of any class including IgG, IgA, IgD, IgE, and IgM, although IgG and IgM will be the most common. Antibody fragments, particularly Fab fragments prepared by

pepsin cleavage, will often be preferable to the use of intact antibody molecules. Methods for preparing antibodies and monoclonal antibodies to particular haptenic or antigenic target substrates are taught in Goding, *Monoclonal Antibodies: Principles and Practice*, Academic Press, 2nd Ed. (1986); Kennett et al. *Monoclonal Antibodies* (1980); U.S. Patent Nos. 4,423,147; 4,381,292; 4,363,799; 4,350,683; and 4,127,124, the disclosures of which are incorporated herein by reference.

The binding site of the polypeptide is defined by a grouping of amino acids which are capable of specifically attaching to the target ligand of interest. The binding site will usually include from about 2 to 15 amino acids, more usually including from about 2 to 10 amino acids, where the amino acids may be contiguous or separated along the primary sequence of the polypeptide. When the amino acids are not contiguous, they will usually be brought together by the folding (secondary structure) of the polypeptide. The binding site will usually define a cavity or other three-dimensional structure which is complementary with a portion of the reactant or reactive intermediate which is being bound. In particular, the side chains of the amino acids within the binding site will interact with the particular structure (referred to as an epitope or determinant site in the case of antibody binding) on the target ligand.

X is an amino acid side chain proximate the binding site on the polypeptide P. The side chain X will normally be located outside of the three-dimensional structure of the binding site, but in some cases may lie within the binding site so long as presence of the active functionality does not substantially interfere with binding between the polypeptide and the target ligand. Generally, however, the side chain X will be located about the periphery of

the binding site at a distance sufficiently close to provide a desired interaction (e.g., drug delivery or quenching of a fluorescent reporter molecule) between the active functionality and the target ligand. The distance between the side chain X and the binding site will usually be less than about 20 Å, more usually being less than about 10 Å, and preferably being less than about 5 Å.

The side chain X may be a natural amino acid side chain (i.e., a side chain of one of the naturally-occurring amino acids) or may be synthetic amino acid side chain (i.e., a side chain of an amino acid which does not occur in nature and which has been specifically introduced to the polypeptide by a synthetic preparatory method as described in more detail hereinbelow). In either case, the amino acid side chain will be sufficiently reactive to allow linking of an active functionality Z through a suitable linking group Y, as described in more detail hereinbelow.

When the side chain X is on a naturally-occurring amino acid, the side chain will usually be non-aliphatic, preferably being selected from the group of the basic side chains (those of lysine, arginine, and histidine), the acidic side chains (aspartate and glutamate), the aliphatic hydroxyl side chains (serine and threonine), thiol (cysteine), sulfide (methionine), phenol (tyrosine), and aminoindole (tryptophan).

Preferably, the amino acid side chain will be present on a relatively rare amino acid chosen to be present proximate the polypeptide binding site, while being substantially absent from the remainder of the exposed surface of the polypeptide. Of course, such rare amino acids may be intentionally introduced to a location proximate the binding site (and deleted from locations away from the binding site) by modification

of the primary structure of the polypeptide by synthetic techniques, as described in more detail hereinbelow. Whether or not an amino acid may be considered rare depends ultimately on the structure of the particular polypeptide. Any amino acid which is exposed to the surface of the polypeptide proximate the binding site, but is substantially absent elsewhere on the surface, can be considered rare. Usually, the rare amino acid will be present at 10 or fewer locations on the polypeptide P surface (other than proximate the binding site), preferably being present at 5 or fewer locations, and more preferably being present at no locations away from the binding site.

In some cases, it will be desirable to introduce unique amino acid side chains by providing synthetic amino acids proximate the binding site of the polypeptide. The use of synthetic amino acids allows the introduction of side chains with chemical reactivities distinct from those of the naturally-occurring amino acid side chains. In this way, localized binding of the active functionalities proximate the binding site can be assured. Particularly suitable synthetic amino acid side chains for covalent attachment of the active functionalities include aryl and alkyl amines (prepared by alkylation of alkyl halides and α -haloketones, by reductive amination of aldehydes, by acylation of isocyanates or isothiocyanates, or by 1,4 additions of α,β unsaturated enones); aldehydes (prepared by reductive amination of alkyl or aryl amines); carboxylic acid derivatives of the form C(O)A, where A = alkyl, aryl, or the like (prepared by transacylation with alkyl or aryl amines); α,β unsaturated enones (prepared by addition reactions with nucleophiles including amines and thiols); and the like.

Y is a linking group selected to provide the necessary covalent bridge between the amino acid side

chain X and a reactive group on the active
functionality Z. Exemplary reactive groups include
thiol, disulfide, ester, aldehyde, α -haloketone,
diazonium, isocyanate, α,β unsaturated ketones and
enones, and the like. The nature of the linking group
is not critical, but it is preferred that the chemistry
used to introduce the linking group be selective for
the desired side chain X in order to avoid linkage of
the active functionality to other amino acid side
chains. The length of the linking group Y is not
critical and may vary between about 1.5 Å and 25 Å,
usually varying between about 5 Å and 12 Å.
Frequently, the linking group will be the result of
reacting the reactive group on the active functionality
with the amino acid side chain, e.g., thioester, azo,
hydroazo, hydroxyl amine, sulfenate, sulfinic acid,
sulfonate, peroxide, anhydride, and the like.
Alternatively, the linking group will be formed from a
bifunctional cross-linking agent. The binding provided
by the linking group should be sufficiently stable to
withstand purification procedures after the binding is
completed as well as withstanding the conditions of
use. In some cases, however, it might be desirable to
provide biologically labile and/or chemically labile
linking groups which will be degraded under preselected
conditions. For example, immunotoxins comprising
toxins coupled to antibodies frequently require that
the toxin be cleavable at the surface of a bound cell
to allow invagination of the toxin. General techniques
for binding molecules to amino acid side chains are
well described in the patent and scientific literature.
See, for example, Cuatrecasas et al. (1969) J. Biol.
Chem. 244:4316; Givol et al. (1971) Biochemistry
10:3461; U.S. Patent Nos. 4,340,535; 4,368,149;
4,489,710; and 4,671,958; and European patent
application 187 658, the disclosures of which are
incorporated herein by reference.

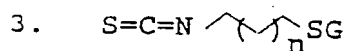
Exemplary linking groups Y may be formed directly between amino acid side chains X and reactive groups on the active functionality Z as follows:

	<u>Linking Group Y</u>	<u>X (or moiety thereon)</u>	<u>Reactive Group on Z</u>
5	$\begin{array}{c} \text{H} \\ \\ -\text{CH}_2-\text{N}-\text{CH}_2 \\ \\ (\text{amine})^2 \end{array}$	aldehyde	amine/ NaBH_3
	$\begin{array}{c} -\text{S}-\text{S}- \\ (\text{disulfide}) \end{array}$	S-SR or SH	SH or S-SR
10	azobenzene	tyrosine	aryldiazonium
	amine	aryl or alkylamine	α -haloketone
	thiourea	aryl or alkylamine	isothiocyanate
15	1,4-Michael adduct	thiol or aryl or alkylamine	α, β -enone
	amide	aryl or alkylamine	anhydroxy-succinimide ester
20	amide	aryl or alkylamide	anhydride

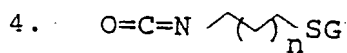
Alternatively, bifunctional cross-linking agents may be used to introduce linking groups Y between the amino acid side chain X and the active functionality Z. Exemplary cross-linking agents are as follows.

	<u>Cross-Linking Agent</u>	<u>Reactive Group on Z</u>
30	1. $\begin{array}{c} \text{O} \\ \\ \text{X}-\text{C}-\text{A} \end{array} \sim \sim \sim \text{SG}$	-OH, -NHR
35	2. $\begin{array}{c} \text{O} \\ \\ \text{H}-\text{C}-\text{A} \end{array} \sim \sim \sim \text{SG/NaCNBH}_3$ to reduce the Schiff's base	-NHR

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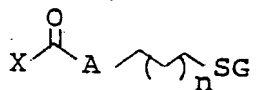
-NHR



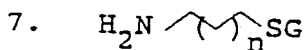
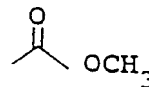
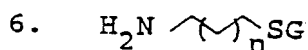
-SH

5

5. Nitration with $\text{HNO}_3/\text{H}_2\text{SO}_4$
followed by reduction with
 NaBH_4 followed by reaction with



10



15

where G is a suitable protecting group, e.g., thiopyridyl or acyl; A is CH_2 , O, or NH; R is aryl or hydrogen; and X is anhydride, anhydroxysuccinimide ester, or other active acyl group.

20

The active functionality Z will be a molecule selected from the group consisting of reporter molecules and chemotherapeutic agents. The size of the active functionality molecule is not critical, but will generally be in the range from about 100 to 2000 daltons (D), more usually being in the range from about 200 to 1000 D. The structure of the active functionality will vary widely, depending on its type and the intended use, but will necessarily include a reactive group which will be available to covalently bind to the polypeptide P either directly or via a bifunctional cross-linking agent. Active functionalities which in their natural state do not include suitable reactant groups will have to be modified to provide for such groups in a manner which does not interfere with their desired activity. Suitable reactive groups on the active functionality are set forth above.

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Reporter molecules and compounds are selected to provide a detectable signal where the strength or other characteristic of the signal may be affected or modulated by binding of the polypeptide to the target ligand (analyte). Suitable reporter molecules include chromogens (e.g., dyes and fluorophores), chemiluminescers, bioluminescers, spin labels, flavins, piezoelectric molecules, biotin, and the like. Numerous suitable reporter molecules are described in U.S. Patent No. 4,366,241, the disclosure of which is incorporated herein by reference.

Suitable chromogens will include molecules and compounds which absorb light in a distinctive range so that a color may be observed, or emit light when irradiated with radiation of a particular wavelength or wavelength range, e.g., fluorescers.

A wide variety of suitable dyes are available, being primarily chosen to provide an intense color with minimum absorption by their surroundings. Illustrative dye types include quinoline dyes, triarylmethane dyes, acridine dyes, alizarin dyes, phthaleins, insect dyes, azo dyes, anthraquinoid dyes, cyanine dyes, phenazathionium dyes, and phenazonium dyes.

A wide variety of fluorescers may be employed either by themselves or in conjunction with quencher molecules. Fluorescers of interest fall into a variety of categories having certain primary functionalities. These primary functionalities include 1- and 2-aminonaphthalene, p,p'-diaminostilbenes, pyrenes, quaternary phenanthridine salts, 9-aminoacridines, p,p'-diaminobenzophenone imines, anthracenes, oxacarbocyanine, merocyanine, 3-aminoequilenin, perylene, bis-benzoxazole, bis-p-oxazolyl benzene, 1,2-benzophenazin, retinol, bis-3-aminopyridinium salts, hellebrigenin, tetracycline, sterophenol, benzimidazolylphenylamine, 2-oxo-3-chromen, indole,

xanthene, 7-hydroxycoumarin, phenoxazine, salicylate, strophanthidin, porphyrins, triarylmethanes and flavin.

Individual fluorescent compounds which have functionalities for linking or which can be modified to incorporate such functionalities include dansyl chloride, fluoresceins such as 3,6-dihydroxy-9-phenylxanthhydryl, rhodamineisothiocyanate, N-phenyl 1-amino-8-sulfonatonaphthalene, N-phenyl 2-amino-6-sulfonatonaphthalene, 4-acetamido-4-isothiocyanato-stilbene-2,2'-disulfonic acid, pyrene-3-sulfonic acid, 2-toluidinonaphthalene-6-sulfonate, N-phenyl, N-methyl 2-aminonaphthalene-6-sulfonate, ethidium bromide, stebrine, auromine-0, 2-(9'-anthroyl)palmitate, dansyl phosphatidylethanolamine, N,N'-dioctadecyl oxacarbocyanine, N,N'-dihexyl oxacarbocyanine, merocyanine, 4-(3'-pyrenyl)butyrate, d-3-aminodesoxy-equilenin, 12-(9'-anthroyl)stearate, 2-methylantracene, 9-vinylanthracene, 2,2'-(vinylene-p-phenylene)bisbenzoxazole, p-bis[2-(4-methyl-5-phenyloxazolyl)]benzene, 6-dimethylamino-1,2-benzophenazin, retinol, bis(3'-aminopyridinium) 1,10-decandiyl diiodide, sulfonaphthylhydrazones of hellibrienin, chlorotetracycline, N-(7-dimethylamino-4-methyl-2-oxo-3-chromenyl) maleimide, N-[p-(2-benzimidazolyl)-phenyl]maleimide, N-(4-fluoranthyl) maleimide, bis(homovanillic acid), resazarin, 4-chloro-7-nitro-2,1,3-benzooxadiazole, merocyanine 540, resorufin, rose bengal, and 2,4-diphenyl-3(2H)-furanone.

Desirably fluorescers should absorb light above about 300 nm, preferably above 350 nm and more preferably above about 400 nm, usually emitting at wavelengths greater than 10 nm higher than the wavelength of the light absorbed. It should be noted that the absorption and emission characteristics of the bound dye may differ from the unbound dye. Therefore, when referring to the various wavelength ranges and characteristics of the dyes, it is intended to indicate

the dyes as employed and not the dye which is unconjugated and characterized in an arbitrary solvent.

Fluorescers are generally preferred to absorptive dyes to the extent that a single fluorescer can provide for multiplication of a signal. By irradiating a fluorescer with light, one can obtain a plurality of emissions. Thus, a single label can provide for a plurality of measurable events.

Detectable signal may also be provided by chemiluminescent and bioluminescent sources. Chemiluminescent sources include a compound which becomes electronically excited by a chemical reaction and may then emit light which serves as the detectible signal or donates energy to a fluorescent acceptor. A diverse number of families of compounds have been found to provide chemiluminescence under a variety of conditions. One family of compounds is 2,3-dihydro-1,4-phthalazinedione. The most popular compound is luminol, which is the 5-amino compound. Other members of the family include the 5-amino-6,7,8-trimethoxy- and the dimethylamino[ca]benz analog. These compounds can be made to luminesce with alkaline hydrogen peroxide or calcium hypochlorite and base. Another family of compounds is the 2,4,5-triphenylimidazoles, with lophine as the common name for the parent product. Chemiluminescent analogs include para-dimethylamino and -methoxy substituents. Chemiluminescence may also be obtained with oxalates, usually oxalyl active esters, e.g., p-nitrophenyl and a peroxide, e.g., hydrogen peroxide, under basic conditions. Alternatively, luciferins may be used in conjunction with luciferase or lucigenins to provide bioluminescence.

Spin labels are provided by reporter molecules with an unpaired electron spin which can be detected by electron spin resonance (ESP) spectroscopy. Exemplary spin labels include organic free radicals,

transition metal complexes, particularly vanadium, copper, iron, and manganese, and the like. An exemplary spin label is nitroxide free radical.

Chemotherapeutic agents will be selected depending on the diseased state which is being treated as well as on the nature of the target ligand. Such agents may be intended to kill diseased cells of the host, kill pathogens, inhibit cellular proliferation, provide hormone therapy, or provide a wide variety of other beneficial interactions between the agent and the target ligand. Exemplary chemotherapeutic agents include toxins, toxin fragments, bactericides, radical scavengers, radical generators, alkylating agents, neurotransmitters, radionuclides, antiviral compounds, antifungal compounds, antineoplastic agents, antimycoplasmal agents, heavy metals, and the like. A list of suitable drugs is provided in Table 1.

TABLE 1

	<u>Drug Type</u>	<u>Examples</u>
20	Antibacterials	Sulfanilamide Polymyxin Chloramphenicol
25		Aminoglycosides: Streptomycin Neomycin Kanamycin Amikacin Gentamicin Tobramycin Streptomycin B Spectinomycin Ampicillin
30	Antivirals	Acyclovir Vir A Symmetrel
	Antifungals	Nystatin
35	Antineoplastics	Adriamycin Cerubidine Bleomycin Alkeran Valban

		Oncovin
		Fluorouracil
	Radiopharmaceuticals	¹²⁵ I
		¹³¹ I
5		^{99m} Tc (Technetium)
	Heavy Metals	Barium
		Gold
		Platinum
	Antimycoplasmids	Tylosine
10		Spectinomycin
	Toxins	Ricin
		Ricin A chain
		Diphtheria toxin
		Diphtheria toxin A chain
		Abrin
		Modeccin

15

20 The polypeptides of the present invention are generally prepared by a two-step technique where the polypeptide (without the active functionality) is first produced and the active functionality is then site-specifically introduced proximate the binding site of the polypeptide. The polypeptides may be obtained from natural sources, such as cell cultures, animal tissues, plant sources, and the like, or may be produced synthetically as described in more detail
 25 hereinbelow. Most commonly, the polypeptides will be antibodies or antibody fragments elicited against the target ligand of interest (or an analog thereof) in a conventional manner.

30 The target ligand of interest may be utilized as an antigen, or if sufficiently small, may be attached to a suitable immunogen for providing antigenicity. Once the antigenic target ligand is obtained, polyclonal antibodies specific for the substrate may be produced by *in vitro* or *in vivo* techniques. *In vitro* techniques involve *in vitro*
 35 exposure of lymphocytes to the antigenic target substrates, while *in vivo* techniques require the

injection of the antigenic target substrates into a wide variety of vertebrates. Suitable vertebrates are non-human, including mice, rats, rabbits, sheep, goats, and the like. Target substrates smaller than about 10 kD, particularly smaller than about 6 kD, will usually be joined to a larger molecule to elicit the desired immune response. With in vivo techniques, the immunogens are injected into the animal according to a predetermined schedule, and the animals are bled periodically with successive bleeds having improved titer and specificity. The injections may be made intramuscularly, intraperitoneally, subcutaneously, or the like, and an adjuvant, such as incomplete Freund's adjuvant, will be employed.

If desired, monoclonal antibodies can be obtained by preparing immortalized cell lines capable of producing antibodies having the desired specificity. Such immortalized cell lines may be produced in a variety of ways. Conveniently, a small vertebrate, such as a mouse, is hyperimmunized with the desired antigen by the method just described. The vertebrate is then killed, usually several days after the final immunization, the animal's spleen removed, and the spleen cells immortalized. The manner of immortalization is not critical. Presently, the most common technique is fusion with a myeloma cell fusion partner, as first described by Kohler and Milstein (1976) Eur. J. Immunol. 6:511-519. Other techniques include EBV transformation, transformation with bare DNA, e.g., oncogenes, retroviruses, etc., or any other method which provides for stable maintenance of the cell line and production of monoclonal antibodies.

When employing fusion with a fusion partner, the manner of fusion is not critical and various techniques may be employed. Conveniently, the spleen cells and myeloma cells are combined in the presence of a non-ionic detergent, usually polyethylene glycol, and

other additives such as Dulbecco's Modified Eagle's medium, for a few minutes. At the end of the fusion, the non-ionic detergent is rapidly removed by washing the cells, the few cells are promptly dispensed in
5 small culture wells (usually in a microtiter plate) at relatively low density, ranging from about 1 to 5×10^5 per well, in a selective medium chosen to support growth of the hybrid cells while being lethal to the myeloma cells. Usually, the myeloma cell line has been
10 mutated to be sensitive to a lethal agent, typically being HAT-sensitive.

After sufficient time, usually from one to two weeks, colonies of hybrids are observed and plates containing hybrid positive wells are identified. The
15 plates and wells having only one colony per well are selected, and supernatants from these wells are tested for binding activity against the target ligand. Once the positive hybridomas are identified, the cell line can be maintained as a viable culture and/or by
20 lyophilization and frozen storage.

Once the hybridomas have been selected, monoclonal antibodies may be isolated from the supernatants of the growing colonies. The yield of antibodies obtained, however, is usually low. The
25 yield may be enhanced by various techniques, such as injection of the hybridoma cell line into the peritoneal cavity of a vertebrate host which accepts the cells. Monoclonal antibodies may then be harvested from the ascites fluid or from the blood.
30 Proteinaceous and other contaminants will usually be removed from the monoclonal antibodies by conventional techniques, e.g., chromatograph, gel filtration, precipitation, extraction, or the like.

Synthetic and recombinant techniques for
35 producing the polypeptides of the present invention will usually start with an exemplary amino acid sequence which is characteristic of a protein which is

known to bind the target ligand with the requisite affinity. Such exemplary amino acid sequences may be derived from enzymes which are known to bind to a target ligand of interest or from antibodies which have been raised against the target substrates as described above. Natural receptors and ligands such as hormones, lymphokines, lectins, avidin (including streptavidin), proteins of the major histocompatibility complex (MHC), T-cell receptors, G proteins, neurotransmitter receptors, DNA binding proteins, e.g., repressors, and the like will also find use in defining binding sites of interest which may be incorporated into synthetic polypeptides.

Synthetic methods may be utilized for preparing polypeptides having fewer than about 200 amino acids, usually having fewer than about 150 amino acids, and more usually having 100 or fewer amino acids. The synthetic preparation methods, as described in more detail below, are particularly convenient for allowing the insertion of synthetic (non-natural) amino acids at a desired location proximate the binding site of the polypeptide, but suffer from low yields and lack of glycosylation when compared to biological synthesis techniques.

Suitable synthetic polypeptide preparation methods may be based on the well-known Merrifield solid-phase synthesis method where amino acids are sequentially added to a growing chain (Merrifield (1963) J. Am. Chem. Soc. 85:2149-2156). Automated systems for synthesizing polypeptides by such techniques are now commercially available from suppliers such as Applied Biosystems, Inc., Foster City, California 94404; New Brunswick Scientific, Edison, New Jersey 08818; and Pharmacia, Inc., Biotechnology Group, Piscataway, New Jersey 08854.

For the preparation of larger and/or glycosylated polypeptides, recombinant preparation

techniques are usually preferred. Such recombinant techniques involve the expression in cultured cells of recombinant DNA molecules encoding the desired polypeptide amino acid sequence. The DNA sequence may itself be synthetic or alternatively be modified from a natural source, i.e., the gene of an exemplary antibody, enzyme, lymphokine, hormone, or other protein. Synthetic DNA sequences (polynucleotides) may be synthesized by well-known techniques. For example, short-single stranded DNA fragments may be prepared by the phosphoramidite method described by Beaucage and Carruthers (1981) Tett. Letters 22:1859-1862. A double-stranded fragment may then be obtained by either synthesizing a complementary strand and annealing the strands together under appropriate conditions or by adding the complementary strand using DNA polymerase with an appropriate primer sequence. Conveniently, automated equipment for preparing the synthetic DNA sequences are available from the suppliers listed above as providing synthetic polypeptide equipment. Alternatively, the desired DNA sequence may be obtained from a suitable cDNA or genomic library obtained from a cell line expressing the exemplary protein of interest. For example, the gene expressing a monoclonal antibody of interest may be isolated from the hybridoma cell line expressing such antibody. The gene may then be modified as described in more detail hereinbelow to substitute or add amino acid(s) capable of binding the active functionality of interest. The techniques for isolating antibody genes from hybridoma cell lines are well described in the scientific literature. See, for example, Gearhart et al. (1983) Proc. Natl. Acad. Sci. USA 80:3439-3443.

The natural or synthetic DNA fragments coding for the desired catalytic or reactive polypeptide will be incorporated in DNA constructs capable of introduction to and expression in an *in vitro* cell

5 culture. The DNA constructs may be suitable for
replication in a unicellular host, such as yeast or
bacteria, but will frequently be intended for
introduction into and integration within the genome of
10 cultured mammalian or other eukaryotic cell lines. DNA
constructs prepared for introduction into bacteria or
yeast will include a replication system recognized by
the host, the DNA fragment encoding the polypeptide of
interest, transcriptional and translation initiation
15 regulatory sequences joined to the 5'-end of the DNA
sequence, and transcriptional and translational
termination regulatory sequences joined at the 3'-end
of the DNA sequence. The transcriptional regulatory
sequences will include a heterologous promoter which is
20 recognized by the host. Conveniently, available
expression vectors which include replication system and
transcriptional and translational regulatory sequences
together with an insertion site for the DNA sequence to
be expressed may be employed.

25 When starting with isolated genes encoding
the exemplary protein, usually an antibody, enzyme,
lymphokine, hormone, or a fragment thereof, it will
usually be desirable to modify the gene to substitute
or add amino acid(s) through which the active
30 functionality will be bound within or near the binding
site. A variety of methods for altering the natural
gene sequence to provide such substitutions or
additions are known and amply described in the patent
and scientific literature. See, for example, *Molecular*
35 *Cloning: A Laboratory Manual* (Maniatis et al., eds.)
Cold Spring Harbor Laboratory, Cold Spring Harbor, New
York (1982) and *Guide to Molecular Cloning Techniques*
(Berger and Kimmel, eds.) *Methods in Enzymology*, Vol.
152, Academic Press, Inc., Orlando, Florida (1987).
Particular techniques for providing such alterations in
the gene include site-directed mutagenesis as described
by Kunkel et al. (1985) *Nucleic Acids Res.* 13:8764 and

cassette mutagenesis as described by Wells et al. (1985) Gene 34:315-323. The altered gene may then be expressed in cell culture as described above and the modified polypeptide obtained by conventional purification techniques.

A second method may be employed for the substitution of synthetic (non-natural) amino acids in an exemplary protein encoded by an unmodified protein gene. The gene is altered, as described above, to replace an amino acid codon at the desired location proximate the binding site with a nonsense codon differing from the gene's termination codon. Conveniently, oligonucleotide-directed mutagenesis can be employed for such substitution. A suppressor tRNA, directed against the nonsense codon, is chemically or enzymatically acylated with the desired synthetic amino acid and added to an *in vitro* transcription/translation system programmed to express the altered DNA sequence. The synthetic amino acid will be inserted at the location of the nonsense codon. The approach, of course, would work with natural amino acids as well, but would be more cumbersome than simple alteration of the DNA sequence to encode the desired natural amino acid.

The desired unique codon must not encode a natural amino acid insertion site (natural tRNA recognition site), and a suitable codon is a TAG termination or amber codon. Any mutation in a gene that generates a termination codon (TAG, TAA, or TGA) leads to premature termination of polypeptide synthesis as a consequence of the inability of the naturally-occurring tRNA to bind and compete with the release factors. Such altered genes will, of course, have to utilize one of the two alternate termination codons. This approach is particularly advantageous in that alternate amino acid substitutions can be obtained

by preparation of different synthetic amino acids at the aminoacylation stage.

Mutations in the anti-codon loop of a number of tRNA molecules leads to an amber suppressor tRNA. See, e.g., Steege and Soll, in: *Biological Regulation and Development*, Vol. I, (Goldberger, ed.) Plenum Press (1978). Amber suppressors, characterized by a 5'-CUA-3' anti-codon loop, no longer respond to codons recognized by their wild-type counterparts, but instead insert amino acids only in response to amber codons (UAG). The desired tRNA carrying a synthetic amino acid can be generated by anti-codon loop replacement, as described by Bruce et al. (1982) *Proc. Natl. Acad. Sci. USA* 79:7127-7131. Alternatively, a gene encoding the tRNA carrying the synthetic amino acid may be synthesized and expressed in a suitable expression system or the suppressor tRNA can be generated by runoff transcription. The chemically or genetically constructed suppressor tRNA must then be aminoacylated with the synthetic amino acid to be incorporated into the polypeptide of the present invention. A suitable chemical acylation method which utilizes N-blocked aminoacyl pCpA dinucleotide synthesized via carbonyl-diimidazole mediated coupling of N-blocked amino acids with protected pCpA-OH, is condensed with an abbreviated tRNA in the presence of T4 RNA ligase. Heckler et al. (1984) *Tetrahedron* 40:87-94. A suitable prokaryotic *in vitro* transcription/translation system is described in Pratt, in: *Transcription and Translation, A Practical Approach* (Hames and Higgs, eds.) IRL Press, Washington (1984).

The recombinant production of antibodies is taught in a number of recent patent applications, including EPO 8430268.0; EPO 85102665.8; EPO 85305604.2; PCT/GB85/00392; EPO 85115311.4; PCT/US86/002269; and Japanese application 85239543, the disclosures of which are incorporated herein by

reference. See also U.S. Patent No. 4,518,584, the disclosure of which is incorporated herein by reference, which describes site-directed mutagenesis of mammalian proteins.

5 Once the polypeptide has been obtained by any of the techniques described above, the active functionality will be covalently attached to an amino acid side chain proximate the binding site of the polypeptide, either directly or through a bifunctional
10 linker as described above. By providing a rare or synthetic amino acid proximate the binding site, the linking group will be localized at the binding site and will be substantially absent from locations on the polypeptide other than proximate the binding site.
15 Alternately, a novel affinity labeling technique may be employed for site-specific introduction of a linking group to the side chains of even common amino acids, as described in more detail hereinbelow.

20 Linking groups and active functionalities may also be introduced to the polypeptides by a novel technique employing cleavable affinity-labeling reagents. Such reagents are obtained by joining cleavable reactive groups to affinity labels, e.g., haptens specific for the protein binding site. By
25 binding the cleavable affinity labels to the protein at the binding site, and subsequently covalently attaching the free end of the reactive group to an amino acid side chain proximate the binding site, the reactive group can be cleaved from the hapten and the hapten
30 removed from the protein, leaving the reactive group in place on the polypeptide. Useful amino acid side chains for attachment of the affinity-labeling reactive groups include carboxylate (aspartate and glutamate), primary amine (lysine), imidazole (histidine), phenol
35 (tyrosine), and thiol (cysteine). The reactive group moiety remaining after cleavage may itself comprise the active functionality, particularly a catalytic or

reactive functionality as defined in greater detail in
co-pending application serial number _____
(attorney docket no. 2307U-255), previously
incorporated herein by reference. More often, however,
5 the reactive group moiety on the polypeptide will be
useful as an attachment site for covalently attaching
the active functionality. The covalent attachment
methods described hereinabove will generally be useful
for attaching functionalities, including reporter
10 molecules and chemotherapeutic agents, to reactive
groups introduced by affinity labeling. Specific
methods for synthesizing such affinity labels and
attaching them to an antibody combining site are taught
in Example 1 in the Experimental section hereinafter.
15 Such cleavable affinity labels are particularly
suitable for selectively modifying polypeptides which
have not been structurally characterized.

The introduction of thiols (useful in forming
disulfide bonds to an active functionality) to the
20 binding site of a polypeptide may be accomplished using
affinity labels as follows. The affinity label may be
a thiol ester or a disulfide reactive group bound to
the hapten, and cleavage of the label after
site-specific derivatization will incorporate a free
25 thiol at the binding site. A thiol ester can be
cleaved with hydroxylamine while a disulfide can be
reduced with dithiothreitol. The disulfides are
reduced under mild conditions chosen to avoid
disulfides buried within the protein. Another
30 functionality suitable for cleavable linkage is acetal,
which would lead to a protein-bound aldehyde moiety
that could be derivatized by reductive amination.
However, the acidic conditions required for cleavage
could lead to inactivation of acid-sensitive proteins.
35 An azobenzene crosslink could be cleaved reductively to
give an aromatic amine, which could then be selectively
derivatized at low pH.

The choice of the reactive group in the affinity-labelling reagents is governed by a number of factors. The reactive linking group should react slowly enough so that it will react primarily at or near the binding site of the protein after equilibrium is established. The group may react with more than one type of amino acid residue, which is advantageous for maximizing the likelihood of successful derivatization. It is disadvantageous in that it can complicate the characterization of the labeled protein. Ideally, a unique residue on the polypeptide should be modified to give a homogeneous adduct. Another important consideration is the synthesis of the labeling reagents. It should be possible to easily vary the length of the reagent in order to optimize incorporation of label into the protein. The affinity label should also be easily synthesized in radiolabeled form, for ease in quantitating label incorporation. Finally, the label should be stable to subsequent protein manipulation conditions, including the degradative methods used in protein sequence determination.

Two preferred reactive groups which may be introduced using affinity labels are diazoketones and aromatic azides. These groups can be transformed into reactive species in the protein binding site by photochemical irradiation and therefore have the advantage that they can be specifically activated in the binding site of a protein. Several other reactive chemical functionalities are also suitable. Diazonium groups react specifically with the phenolic side chain of tyrosine. Epoxide groups react with nucleophilic side chains including primary amines (lysine) and carboxylates (aspartate and glutamate). Aldehydes react with the primary amine side chain of lysine to form Schiff base intermediates which can be reduced with sodium cyanoborohydride. The use of tritiated

NaCNB³H₃ leads to tritium incorporation, facilitating the characterization of the labeled protein. Finally, α -bromoketones are versatile affinity-labeling reagents. Their reactivity is sufficiently slow that they can label proteins nearly stoichiometrically in their binding sites. Moreover, they react with a number of nucleophilic side chains, including carboxylate, primary amine (lysine), imidazole (histidine), and phenol (tyrosine).

The polypeptides of the present invention having attached reporter molecules will be useful in performing assays for analytes in biological and other samples. In general, the assays will be useful for detecting the presence of the target ligands of the polypeptide, i.e., the analyte will be the target ligand. Presence of the target ligand in a sample may be detected and optionally quantitated based on an observed change in the signal provided by the reporter molecule, usually a reduction in the strength of the signal caused by an interaction between the reporter molecule and the bound target ligand. Thus, the observed signal will be inversely proportional to the concentration or amount of target ligand present in the sample. In specific systems, the signal may be calibrated in order to provide quantitative or semi-quantitative measurement of a desired analyte.

The compositions of the present invention will be particularly advantageous in that they allow the performance of non-competitive, homogeneous assays. As the polypeptides can directly bind the target ligand and provide a signal which is inversely proportional to the concentration of target ligand, no ligand displacement is necessary. Moreover, no separation step is required as the signal modulation can be achieved in the sample medium.

The present invention also allows for the use of secondary binding systems in the performance of

assays. By attaching intermediate binding species, e.g., biotin, a hapten, or the like, to the polypeptide proximate the binding site, the reporter molecule can be introduced to the system through a binding protein specific for the intermediate binding species, e.g., avidin or antibody specific for the hapten. Thus, biotin and haptens are considered reporter molecules for the purpose of the present invention.

The polypeptides of the present invention having attached chemotherapeutic agents will be useful in providing therapeutic treatment of humans and other animals. In particular, polypeptides bound to cellular toxins may be used to treat different forms of cancer where the neoplastic cells are characterized by a unique tumor antigen which allows specific attachment of the polypeptides, usually through an antibody directed against the tumor antigen. Additionally, bacterial infection may be treated by antibodies specific for the bacteria bound to a suitable bactericide, such as an antibiotic.

The polypeptides of the present invention having bound chemotherapeutic agents may be incorporated in conventional pharmaceutical formulations for administration to humans and other animals. Such pharmaceutical compositions should contain a therapeutic dosage of the polypeptide in a suitable pharmaceutically-effective carrier. The pharmaceutical carrier may be any compatible, non-toxic substance suitable to deliver the polypeptides to the patient. Sterile water, alcohols, fats, waxes, and inert solids may be used as a carrier. Pharmaceutically-acceptable adjuvants, buffering agents, dispersing agents, and the like, may also be incorporated in the pharmaceutical compositions of the present invention. Such pharmaceutical compositions may contain a single polypeptide according to the present invention, or may contain two or more such

polypeptides. Additionally, other therapeutically active ingredients may be included. Such "cocktails" including two or more polypeptides and/or active ingredients having different specificities and/or different therapeutic activities may be beneficial under a variety of circumstances.

The pharmaceutical compositions of the present invention will be suitable for oral or parenteral administration, particularly for parenteral administration, including subcutaneous, intramuscular, and intravenous injection. A typical pharmaceutical composition for intramuscular injection would contain 1 ml sterile buffered water and 50 mg of the polypeptide composition of the present invention. Typical composition for intravenous and fusion would contain about 250 ml of sterile Ringer's solution, and 150 mg of monoclonal antibody. Actual methods for preparing parenterally administratable compositions are known to those skilled in the art and are amply described in the patent, medical and scientific literature. Specific reference is made to *Remington's Pharmaceutical Science*, 15th Ed., Mack Publishing Company, Easton, Pennsylvania (1980), the disclosure of which is incorporated herein by reference. Polypeptides of the present invention can be lyophilized for storage and reconstituted in a suitable carrier prior to use. This technique has been shown to be effective with a wide variety of antibodies and other proteins. It will be appreciated by those skilled in the art that lyophilization and reconstitution can lead to varying degrees of antibody activity loss and that the amount of the antibodies or other polypeptides in the pharmaceutical compositions may have to be adjusted to compensate for such loss.

The following examples are offered by way of illustration, not by way of limitation.

EXPERIMENTAL

1. Introduction of Active Functionality to Antibody Using Affinity Label.

5 The following studies were carried out on the IgA MOPC315, which binds substituted 2,4-dinitrophenyl (DNP) ligands with association constants ranging from 5×10^4 to 10^6 M^{-1} (Haselkorn et al. (1974) Biochemistry 13:2210). Although a three-dimensional structure is not available, the antibody combining site
10 has been characterized by spectroscopic methods (UV, fluorometry, NMR), chemical modification, and amino acid sequencing of the variable region. Moreover, earlier affinity-labeling studies with reagents of varying structures (Givol and Wilcheck (1977) Meth.
15 Enzy. 46:479; Givol et al. (1971) Biochemistry 10:3461; and Strausbauch et al. (1971) Biochemistry 10:4342) defined a number of reactive amino acid side chains in the vicinity of the combining site.

Cleavable tethers were incorporated into
20 affinity labels specific for the MOPC315 combining site, as shown in Fig. 1. These affinity-labeling reagents contain the DNP group linked to electrophilic aldehyde or α -bromoketone groups via cleavable disulfide or thiophenyl linkages. Covalent attachment
25 of the label to the antibody, followed by cleavage of the crosslink and removal of the free ligand, results in site-specific incorporation of a free thiol into the antibody combining site. The geometry of affinity-labeling reagents 1-5 varies with regard to
30 the distance between the DNP group and electrophilic moiety since the position of a nucleophilic lysine, histidine, or tyrosine side chain is not precisely known. The syntheses of affinity labels 1-5 are outlined in Fig. 2, and are described in detail
35 hereinbelow.

(N-2,4-Dinitrophenyl)-2-aminoethyl
2-(1,3-dioxolanyl)-ethyl disulfide 6.

Affinity labels 1 and 2 were prepared as follows:

5 To a suspension of N,N'-bis-(2,4-dinitrophenyl)-cystamine (Chan et al. (1979) Phosphorous Sulfur 7:41) (4.84 g, 10 millimoles) in dimethylformamide (200 ml) with triethylamine (50 μ l) was added 2-(2-mercaptoethyl)-1,3-dioxolane (2.45 g, 18
10 millimoles), and the mixture was stirred at 60° C for 24 hours. Hydrogen peroxide (2 ml of a 30% solution) was added and the dimethylformamide was removed in vacuo.

15 The residual oil was dissolved in methylene chloride (100 ml). The methylene chloride layer was washed with water (3 x 5 ml), dried over MgSO_4 , and concentrated in vacuo to an oil. Silica gel column chromatography with 3:2 hexanes: ethyl acetate (R_f -0.31) afforded 6 (1.37 g, 3.65 millimoles, 20%) as
20 an orange oil. IR (thin film): 3353, 2923, 1623, 1525, 1342, 1138 cm^{-1} ; $^1\text{H-NMR}$, CDCl_3 : δ 2.08 (m, 2H), 2.82 (t, 2H, J = 7.1 Hz), 3.00 (t, 2H, J = 6.6 Hz), 3.8 -4.0 (m, 6H), 4.95 (t, 1H, J = 4.3 Hz), 7.00 (d, 1H, J = 9.5
25 Hz), 8.30 (d, 1H, J = 9.6 Hz), 8.80 (s, 1H), 9.16 (d, 1H, J = 2.6 Hz); mass spectrum (EI) 375 (M+). Anal. Calcd. for $\text{C}_{13}\text{H}_{17}\text{N}_3\text{O}_6\text{S}_2$: C, 41.60; H, 4.53; N, 11.20; S, 17.07. Found: C, 41.57; H, 4.67; N, 11.10; S, 16.99.

30 (N-2,4-Dinitrophenyl)-2-aminoethyl
3-oxopropyl disulfide 1.

A solution of dioxolane 6 (0.50 g, 1.33 millimoles) in water (2 ml), acetonitrile (2 ml) and acetic acid (8 ml) was heated at reflux for 20 h. At
35 this time the mixture was cooled to room temperature and added slowly to 150 ml of cold saturated aqueous NaHCO_3 . The aqueous layer was extracted with methylene chloride (3 x 50 ml). The combined extracts were dried

over MgSO_4 and concentrated *in vacuo* to an orange oil. Silica gel chromatography using a gradient of 30 to 40% ethyl acetate in hexanes afforded 1 (0.30 g, 0.91 millimole, 68%) as an orange semi-solid: $R_f = 0.22$ in 3:2 hexanes: ethyl acetate; IR (KBr pellet) 3353, 1722, 1623, 1525, 1504, 1426, 1342, 1131 cm^{-1} ; $^1\text{H-NMR}$ (CDCl_3) δ 2.90-3.04 (m, 6H), 3.79 (t, 2H, $J = 6.5$ Hz), 7.01 (d, 1H, $J = 9.5$ Hz), 8.31 (d, 1H, $J = 9.5$ Hz), 8.80 (m, 1H), 9.15 (d, 1H, $J = 2.6$ Hz) 9.83 (s, 1H); mass spectrum (EI) 331 (M⁺). Anal. Calcd. for $\text{C}_{11}\text{H}_{13}\text{N}_3\text{O}_5\text{S}_2$: C, 39.88; H, 3.93; N, 12.69; S, 19.34. Found: C, 40.01; H, 3.91; N, 12.66; S, 19.34.

15 (N-2,4-Dinitrophenyl)-2-aminoethyl
3-(1,3-dioxolanyl)-propyl disulfide 7.

This compound was prepared as described above for 6, starting with N,N'-bis-(2,4-dinitrophenyl)-cystamine (2.42 g, 5 millimoles) and 3-(3-mercaptopropyl)-1,3-dioxolane (1.48 g, 10 millimoles), to give 7 (0.60 g, 1.54 millimoles, 15%) as an orange oil: $R_f = 0.43$ in 3:2 hexanes: ethyl acetate; IR (thin film) 3360, 2923, 1623, 1592, 1528, 1426, 1335, 1131 cm^{-1} ; $^1\text{H-NMR}$ (CDCl_3); δ 1.80-1.95 (m, 6h), 2.78 (t, 2H, $J = 7.0$ Hz), 2.99 (t, 2H, $J = 6.7$ Hz), 3.8-4.0 (m, 6H), 4.87 (t, 1H, $J = 4.2$ Hz), 7.01 (d, 1H, $J = 9.5$ Hz), 8.32 (d, 1H, $J = 9.5$ Hz), 8.79 (s, 1H), 9.16 (d, 1H, $J = 2.6$ Hz); mass spectrum (EI) 389 (M), 368, 359, 294, 279, 256, 225, 210, 196. Anal. Calcd. for $\text{C}_{14}\text{H}_{19}\text{N}_3\text{O}_6\text{S}_2$: C, 43.19; H, 4.88; N, 10.80; S, 16.45. Found: C, 43.27; H, 4.65; N, 10.79; S, 16.40.

35 (N-2,4-Dinitrophenyl)-2-aminoethyl
4-oxobutyl disulfide 2.

This compound was prepared as described above for 1, starting with dioxolane 6 (389 mg, 1.0 millimole), to give 2 (340 mg, 0.99 millimole, 99%) as an orange solid: mp 64-65° C; $R_f = 0.25$ in 3:2 hexanes:

ethyl acetate; IR (KBr pellet) 3331, 3092, 2923, 2846, 1722, 1630, 1589, 1525, 1415, 1342, 1247, 1146 cm^{-1} ; $^1\text{H-NMR}$ (CDCl_3) δ 2.06 (t, 2H, $J = 7.3$ Hz), 2.63 (t, 2H, $J = 7.0$ Hz), 2.75 (t, 2H, $J = 7.1$ Hz), 3.00 (t, 2H, $J = 6.6$ Hz), 3.81 (t, 2H, $J = 6.1$ Hz), 7.01 (d, 1H, $J = 9.5$ Hz), 8.31 (d, 1H, $J = 9.5$ Hz), 8.79 (s, 1H), 9.16 (d, 1H, $J = 2.6$ Hz), 9.80 (s, 1H); mass spectrum (EI) 345 (M). Anal. Calcd. for $\text{C}_{12}\text{H}_{15}\text{N}_3\text{O}_5\text{S}_2$: C, 41.74; H, 4.35; N, 12.17; S, 18.55. Found: C, 41.71; H, 4.38; N, 12.11; S, 18.49.

Affinity labels 3, 4, and 5 were prepared as follows:

3-(S-DNP)-mercaptopropanoic acid 8.

To a solution of 3-mercaptopropanoic acid (1.59 g, 15 millimoles) in 70 ml of 2.0 M aqueous sodium acetate (pH 5.2) was added a solution of 2,4-dinitrofluorobenzene (3.32 g, 18.0 millimoles) in 50 ml of absolute ethanol over 15 min with stirring at room temperature under nitrogen. After 24 hours, the pale yellow solid precipitate was collected and washed with water to give 8 (3.40 g, 12.5 millimoles, 83%): mp 154.5 -157.5° C; IR (KBr) 3630, 3476 (br), 3113, 3082, 1718, 1589, 1511, 1344, 1055, 922 cm^{-1} ; $^1\text{H-NMR}$ (acetone- d_6) δ 2.71 (t, 2H, $J = 7.5$ Hz), 3.40 (t, 2H, $J = 7.5$ Hz), 3.40 (t, 2H, $J = 7.4$ Hz), 3.40 (s, 1H), 7.87 (d, 1H, $J = 9.1$ Hz), 8.32 (dd, 1H, $J = 2.5, 9.0$ Hz), 8.76 (d, 1H, $J = 2.5$ Hz); mass spectrum (FAB^-) 271 (M-H) $^-$, 249, 199, 183, 141, 113, 109. Anal. Calcd. for $\text{C}_9\text{H}_8\text{N}_2\text{O}_6\text{S}$: C, 39.71; H, 2.96; N, 10.29; S, 11.78. Found: C, 39.92; H, 2.96; N, 10.15; S, 11.64.

To a solution of 8 (0.70 g, 2.5 millimoles) in dry dioxane (10 ml) was added thionyl chloride (5 ml). The mixture was stirred under nitrogen for 12 hours. The volatiles were removed in vacuo and the yellow residue was dissolved in dry dioxane (25 ml). A solution of diazomethane (0.6 M) in diethyl ether (25

ml) was added. The mixture was stirred at 0° C for 4 hours after which the volatiles were removed *in vacuo*. The residue was purified by silica gel chromatography using 9:1 methylene chloride: ethyl acetate as the eluent to give 9 (0.29 g, 0.92 millimoles, 36%) as a yellow solid: mp 117-119°C; R_f = 0.52 in above eluent; IR (KBr pellet) 3458 (br), 3090, 2111, 1637, 1583, 1508, 1340, 1096 cm^{-1} ; $^1\text{H-NMR}$ (CDCl_3) δ 2.75 (t, 2H, J = 7.1 Hz), 3.35 (t, 2H, J = 7.3 Hz), 5.32 (s, 1H), 7.61 (d, 1H, J = 9.0 Hz), 8.48 (dd, 1H, J = 2.5, 9.0 Hz), 9.07 (d, 1H, J = 2.5 Hz); mass spectrum (FAB^-) 296 (M^-), 199, 183, 141, 109. Anal. Calcd. for $\text{C}_{10}\text{H}_8\text{N}_4\text{O}_5\text{S}$: C, 40.54; H, 2.72; N, 18.91; S, 10.82. Found: C, 40.81, H, 2.89; N, 18.59; S, 10.79.

1-Bromo-2-oxo-4-(S-DNP)-mercaptobutane 3.

To a solution of 9 (95 mg, 0.32 millimoles) in dry dioxane (5 ml) at 25° C under nitrogen was added a saturated solution of HBr in dioxane (2 ml). The mixture was stirred for 10 minutes at which time the volatiles were removed completely *in vacuo* to give pure 3 (130 mg, 0.373 millimoles, 86%) as a yellow solid: mp 99-101° C; R_f = 0.78 in 9:1 methylene chloride: ethyl acetate; IR (KBr) 3106, 3097, 3012, 2958, 1724, 1592, 1510, 1339, 1068, 1053 cm^{-1} ; $^1\text{H-NMR}$ (acetone-d_6) δ 3.24 (t, 2H, J = 7.0 Hz), 3.46 (t, 2H, J = 7.0 Hz), 4.28 (s, 2H), 7.98 (d, 1H, J = 9.0 Hz), 8.48 (dd, 1H, J = 2.5, 9.0 Hz); mass spectrum (FAB^-) 268 ($\text{M} - \text{HBr}$) $^-$, 233, 199, 183, 109. Anal. Calcd. for $\text{C}_{10}\text{H}_9\text{BrN}_2\text{O}_5\text{S}$: C, 34.40; H, 2.60; Br, 22.89; N, 8.02; S, 9.18. Found C, 34.50; H, 2.60; Br, 22.70; N, 7.88; S, 9.04.

4-(S-DNP)-mercaptobutanoic acid 10.

This compound was prepared as described above for 8, starting with 4-mercaptobutanoic acid (1.80 g, 15 millimoles) giving 10 (3.21 g, 11.2 millimoles, 75 %) as a pale yellow solid: mp 126.5-130° C; IR (KBr)

3528 (br), 3117, 3090, 2948, 1709, 1587, 1518, 1341 cm^{-1} ; ^1H - NMR (DMSO-d_6) δ 1.85 (m, 2H), 2.34 (t, 2H, $J = 7.0$ Hz), 3.18 (t, 2H, $J = 7.5$ Hz), 3.78 (s, 1H), 7.95 (d, 1H, $J = 9.1$ Hz), 8.41 (dd, 1H, $J = 2.4, 9.0$ Hz), 8.84 (d, 1H, $J = 2.4$ Hz); mass spectrum (FAB^-) 285 ($\text{M} - \text{H}$) $^-$, 249, 199, 139. Anal. Calcd. for $\text{C}_{10}\text{H}_{10}\text{N}_2\text{O}_6\text{S}$: C, 41.96; H, 3.52; N, 9.79; S, 11.20. Found: C, 41.94; H, 3.50; N, 9.60; S, 10.97.

1-Diazo-2-oxo-5-(S-DNP)-mercaptopentane 11.

This compound was prepared as described above for 9, starting with 10 (1.00 g, 3.49 millimoles), to give 11 (0.68 g, 2.20 millimoles, 63%) as a yellow solid: mp 84-86° C; $R_f = 0.52$ in 9:1 methylene chloride: ethyl acetate; IR (KBr) 3106, 2939, 2866, 2104, 1626, 1589, 1513, 1343 cm^{-1} ; ^1H - NMR (CDCl_3) δ 2.10 (m, 2H), 2.55 (t, 2H, $J = 5.7$ Hz), 3.10 (t, 2H, $J = 7.5$ Hz), 5.29 (s, 1H), 7.74 (d, 1H, $J = 9.0$ Hz), 8.38 (dd, 1H, $J = 2.5, 9.0$ Hz), 9.06 (d, 1H, $J = 2.4$ Hz); mass spectrum (FAB^-) 310 (M^-), 249, 199, 139, 107. Anal. Calcd. for $\text{C}_{11}\text{H}_{10}\text{N}_4\text{O}_5\text{S}$: C, 42.58; H, 3.25; N, 18.06; S, 10.33. Found: C, 42.79; H, 3.39; N, 17.93; S, 10.14.

1-Bromo-2-oxo-5-(S-DNP)-mercaptopentane 4.

This compound was prepared as described above for 3, starting with 11 (101 mg, 0.326 millimoles) to give 4 (116 mg, 0.32 millimoles, 98%) as a yellow solid: mp 87-90° C; $R_f = 0.78$ in 9:1 methylene chloride: ethyl acetate; IR (KBr) 3113, 3009, 2953, 1723, 1592, 1512, 1343 cm^{-1} ; ^1H - NMR (DMSO-d_6) δ 1.89 (m, 2H), 2.79 (t, 2H, $J = 7.1$ Hz), 3.17 (t, 2H, $J = 7.4$ Hz), 4.36 (s, 2H), 7.88 (d, 1H, $J = 9.1$ Hz), 8.43 (dd, 1H, $J = 2.5, 9.0$ Hz), 8.85 (d, 1H, $J = 2.6$ Hz); Anal. Calcd. for $\text{C}_{11}\text{H}_{11}\text{BrN}_2\text{O}_5\text{S}$: C, 36.38; H, 3.05; Br, 2.00; N, 7.71. S, 8.83. Found: C, 36.57; H, 3.17; Br, 21.83; N, 7.49; S, 9.01.

5-(S-DNP)-mercaptopentanoic acid 12.

This compound was prepared as described above for 8, starting with 4-mercaptopentanoic acid (2.00 g, 15 millimoles) giving 12 (4.20 g, 14.0 millimoles, 93%) as a pale yellow solid: mp 159 -161° C; IR (KBr) 3117, 2948, (br), 1707, 1587, 1587, 1517, 1341 cm^{-1} ; ^1H - NMR (acetone- d_6) δ 1.83 (m, 4H), 2.39 (t, 2H, $J = 6.8$ Hz), 2.83 (s, 1H) 3.26 (t, 2H, $J = 7.0$ Hz), 7.95 (d, 1H, $J = 9.0$ Hz), 8.45 (dd, 1H, $J = 2.5, 9.0$ Hz), 8.96 (d, 1H, $J = 2.5$ Hz); mass spectrum (FAB $^-$) 299 (M-H) $^-$, 199, 183, 141, 113, 109. Anal. Calcd. for $\text{C}_{11}\text{H}_{12}\text{N}_2\text{O}_6\text{S}$: C, 44.00; H, 4.03; N, 9.33. S, 10.68. Found: C, 43.83; H, 3.92; N, 9.42; S, 10.59.

1-Diazo-2-oxo-6-(S-DNP)-mercaptohexane 13.

This compound was prepared as described above for 9, starting with 12 (1.00 g, 3.33 millimoles), to give 13 (0.70 g, 2.15 millimoles, 65%) as a yellow solid: mp 120-122° C; $R_f = 0.50$ in 9:1 methylene chloride: ethyl acetate; IR (KBr) 3093, 2937, 2110, 1637, 1584, 1508, 1336, 1109 cm^{-1} ; ^1H - NMR (CDCl_3) δ 1.83 (m, 4H), 2.39 (t, 2H, $J = 5.8$ Hz), 3.04 (t, 2H, $J = 7.4$ Hz), 5.25 (s, 1H), 7.53 (d, 1H, $J = 9.0$ Hz), 8.37 (dd, 1H, $J = 2.5, 9.0$ Hz), 9.07 (d, 1H, $J = 2.5$ Hz); mass spectrum (FAB $^-$) 324, 307, 267, 199, 183, 141, 109. Anal. Calcd. for $\text{C}_{12}\text{H}_{12}\text{N}_4\text{O}_5\text{S}$: C, 44.44; H, 3.73; N, 17.28. S, 9.89. Found: C, 44.60; H, 3.74; N, 16.99; S, 9.65.

1-Bromo-2-oxo-6-(S-DNP)-mercaptohexane 5.

This compound was prepared as described above for 3, starting with 13 (119 mg, 0.366 millimoles) to give 5 (0.127 g, 0.336 millimoles, 92%) as a yellow solid: mp 80-83°C; $R_f = 0.78$ in 9: 1 methylene chloride: ethyl acetate; IR (KBr) 3121, 2937, 2872, 1719, 1586, 1513, 1339, 1095, 1053; ^1H -NMR (acetone- d_6) δ 1.81 (m, 4H), 2.79 (t, 2H, $J = 6.6$ Hz), 3.24 (t, 2H, $J = 6.8$ Hz), 4.18

(s, 2H), 7.93 (d, 1H, J=9.0Hz), 8.45 (dd, 1H, J=2.5, 9.0Hz), 8.95 (d, 1H, J=2.5Hz); mass spectrum (FAB⁻) 378 (M⁻), 307, 263, 199, 183, 141, 109. Anal. Calcd. for C₁₂H₁₃BrN₂O₅S: C, 38.21; H, 3.47; Br, 21.18; N, 7.43; S, 8.50. Found: C, 38.40; H, 3.45; Br, 21.34; N, 7.34; S, 8.46.

Antibody Modification

Affinity Labeling.

The strategy for introducing a thiol into the binding site of MOPC315 is outlined in Fig. 3. These steps were carried out with the Fab fragment, generated by treating the reduced and alkylated IgA with papain followed by affinity chromatography on DNP-coupled Sepharose 4B (Goetzl (1970) Biochemistry 9:1267). The Fab fragment (at 10 μ M) was treated with either 1.5 equivalents of aldehydes 1 or 2 for one hour followed by 7.5 equivalents of NaCNBH₃ in 0.2 M sodium phosphate; pH 7.0, at 37°C for 20 hours, or with 1.3 equivalents of α -bromoketones 3, 4, or 5 in 0.1 M sodium bicarbonate, pH 9.0; at 37°C for 16 hours. In both cases, the labeled Fab was purified by chromatography on Sephadex G-50 in 0.1 M sodium phosphate, pH 7.3, and, after lyophilization, subsequent affinity chromatography on DNP-coupled Sepharose 4B (Goetzl, supra.) to remove unlabeled or non-specifically labeled Fab. The extent of derivatization was quantitated spectrophotometrically (for labels 1 and 2, λ_{max} =360nm, ϵ =12,800M⁻¹ cm⁻¹) and additionally, for labels 1 and 2, by incorporation of tritium from NaCNB³H₃ (using 12 mCi/millimole NaCNB³H₃). With labels 2 and 4, over 90% of the label was incorporated (85% yield after affinity chromatography) (Table 2). In both cases, less than 10% non-specific labeling occurred in the presence of 10 mM of the competitive inhibitor DNP-glycine.

Table 2

Incorporation of Affinity Labels into Fab

5	compd	% Fab labeled (without DNP-glycine)	% Fab labeled (with DNP-glycine)
	1	22	5
	2	95	10
	3	0	0
10	4	85	0
	5	15	0

15 Cleavage of Labels and Isolation
of Stable S-Thiopyridyl Adducts.

20 Cleavage of the Fab affinity-labeled with 2
or 4 (2.0mg) with 50 mM dithiothreitol in 2 mM EDTA,
0.1 M sodium phosphate, pH 8.0 (2 ml) for 12 hours at
37°C afforded the free thiol. The thiol-containing Fab
was collected by fast-desalting chromatography using a
Pharmacia FPLC column in 0.1 M sodium phosphate, pH
7.3, directly into 1.0 ml of a 4.5 mM solution of
2,2'-dithiodipyridine (0.1 M sodium phosphate, pH 5.5,
containing 15% acetonitrile). This mixture (final
25 volume of 10 ml) was allowed to react at 20°C for 12
hours after which excess 2,2'-dithiodipyridine was
removed by exhaustive dialysis. The thiolated antibody
was derivatized in greater than 90% yield (65% recovery
in the case of 2) based on the absorbance of
30 thiopyridine at 343 nm ($\epsilon=7060 \text{ M}^{-1} \text{ cm}^{-1}$) (Grasseti et
al. (1967) Arch. Biochem. Biophys. 119:41) after
cleavage with 10 mM dithiothreitol, and the protein
absorbance at 280 nm ($E_{0.1\%}=1.37$, MW=50,000 for Fab).
Determination of Modified Site.

35 Fab fragments labeled with 2 or 4 were
subjected to tryptic digestion and peptide mapping in
order to determine the selectivity of thiol

incorporation. Fab was affinity-labeled in the presence of label 2 and NaCNB^3H_3 (12 mCi/millimole) or with ^3H -labeled 4 (4 mCi/millimole) as described above. ^3H -labeled 4 was prepared as described for 4, starting with 3,5- ^3H -2,4-dinitrofluorobenzene. The labeled Fab (1.0 mg/ml) was then denatured in 8 M urea, 0.1 M Tris-HCl, pH 8.0, reduced with 20 mM dithiothreitol (1 hour at 37°C) and alkylated with 60 mM Tris-HCl, pH 8.0, reduced with 20 mM dithiothreitol (1 hour at 37°C) and alkylated with 60 mM iodoacetamide (1 hour at 37°C). After dialysis against 7 M urea, 20 mM Tris-HCl, pH 8.0, the heavy and light chains were separated by anion exchange chromatography on a Pharmacia Mono Q FPLC column. Separation was achieved at 1.0 ml/min in 7 M urea, 20 mM Tris-HCl, pH 8.0, with a linear gradient of 40 mM to 200 mM sodium chloride over 30 min.

In the case of Fab labeled with 2, the heavy chain was found to contain over 95% of the incorporated tritium; with Fab labeled with 4, over 95% of the tritium label was on the light chain. In each case, the radiolabeled chain was dialyzed against 2 M urea/100 mM ammonium bicarbonate, pH 8.2, and then treated with trypsin (1:25 w/w trypsin:protein) in the presence of 0.1 mM CaCl_2 at 37°C in the dark. After 8 hours, the reactions were quenched with 10% v/v acetic acid. The radio-labeled peptides were purified by reverse phase HPLC with a 70 min. linear gradient from 0 to 50% acetonitrile in water at 1.0 ml/min; (0.1% and 0.06% (v/v) trifluoroacetic acid was added to the water and acetonitrile, respectively.) Peptides were detected by their absorbance at 214 nm, fractions were collected at 1.0 ml intervals, and aliquots were counted for radioactivity (Fig. 4). Fractions containing radioactivity were re-chromatographed using a 70 min. linear gradient from 0 to 50% 2-propanol in water at 0.75 ml/min. The water and the 2-propanol

contained 0.1% and 0.06% trifluoroacetic acid, respectively.

The amino acid sequences of the pure peptides were determined on an Applied Biosystems 477A Protein Sequencer. With heavy chain obtained from Fab labeled with 2, all of the detectable derivatization was on leucine 52H. With the light chain from Fab labeled with 4, all of the detectable derivatization was on tyrosine 34L. These residues are identical to those labeled by Givol and co-workers (Haimovich et al. (1972) Biochemistry 11:2389) with the reagents bromoacetyl-N^ε-DNP-L-lysine and N-bromoacetyl-N'-DNP-ethylenediamine.

Ester Cleavage Using Thiol-Derivatized Antibody.

In addition to modifying the thiolated antibody selectively with catalytic and other groups, the thiol itself can act as a nucleophile in the thiolysis of appropriate substrate. DNP-containing esters 14 and 15 (Fig. 5) were chosen as substrates for the reaction with thiolated Fab labeled with 2 or 4, respectively. The position of the cleavable linkage in affinity label 2 approximates that of the ester in the corresponding substrate 14, ensuring that the thiol is positioned appropriately in the combining site to attack the ester. Likewise, the thiol in Fab labeled with 4 should be positioned to attach ester 15. Moreover, these substrates contain fluorescent coumarin leaving groups, which can readily be detected at nanomolar concentrations.

Synthesis of Substrates.

(N-DNP)-3-Aminopropanoic acid
7-hydroxycoumarin ester 14.

A mixture of N-DNP-3-aminopropanoic acid (1.28g, 5 millimoles) and 7-hydroxycoumarin (0.81 g, 5 millimoles) in phosphorus oxychloride (8 ml) was heated

at reflux under nitrogen for 2 hours. The mixture was cooled to room temperature and added to 60 ml of cold water. The brown solid was filtered, washed with cold water and dried in vacuo. Trituration with acetone gave 14 (0.79 g, 2.0 millimoles, 40%) as a light brown solid; mp 155-156°C, IR (KBr pellet) 3374, 3107, 2368, 1750, 1722, 1624, 1532, 1426, 1349, 1159, 1127 cm^{-1} ; $^1\text{H-NMR}$ (DMSO-d_6) δ 3.04 (t, 2H, $J=6.7\text{Hz}$), 3.90 (t, 2H, $J=6.5\text{Hz}$), 6.48 (d, 1H, $J=9.6\text{Hz}$), 7.17 (d, 1H, $J=8.4\text{Hz}$), 7.30 (s, 1H), 7.37 (d, 1H, $J=9.7\text{Hz}$), 7.78 (d, 1H, $J=8.5\text{Hz}$), 8.07 (d, 1H, $J=9.3\text{Hz}$), 8.29 (d, 1H, $J=9.6\text{Hz}$), 8.87 (s, 1H), 8.99 (m, 1H); mass spectrum (FAB^+) 400(MH^+). Anal. Calcd. for $\text{C}_{18}\text{H}_{13}\text{N}_3\text{O}_8$: C, 54.14; H, 3.26; N, 10.53. Found: C, 54.05; H, 3.26; N, 10.23.

2,4-Dinitrobenzoic acid, 7-hydroxycoumarin ester 15.

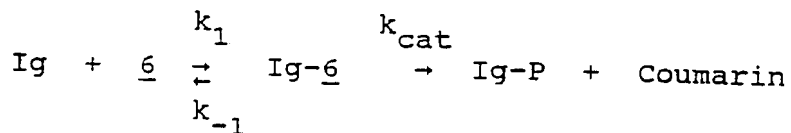
A mixture of 2,4-dinitrobenzoic acid (1.06 g, 5.0 millimoles) and 7-hydroxycoumarin (0.81 g, 5.0 millimoles) in phosphorus oxychloride (4 ml) was heated at reflux with a calcium sulfate drying tube for 40 min. The mixture was cooled to room temperature and added to water (40 ml). The brown solid was filtered, washed with cold water and dried in vacuo to give 15 (1.32 g, 3.7 millimoles, 74%) as a red-brown solid: mp 200-205°C; IR (KBr) 3107, 3057, 2364, 1736, 1619, 1544, 1348, 1246, 1122 cm^{-1} ; $^1\text{H-NMR}$ (DMSO-d_6) δ 6.53 (d, 1H, $J=9.6\text{Hz}$), 7.34 (dd, 1H, $J=2.2, 8.5\text{Hz}$), 7.47 (d, 1H, $J=2.1\text{Hz}$), 7.89 (d, 1H, $J=8.5\text{Hz}$), 8.12 (d, 1H, $J=9.6\text{Hz}$), 8.44 (d, 1H, $J=8.4\text{Hz}$), 8.78 (dd, 1H, $J=2.2, 8.4\text{Hz}$), 8.93 (d, 1H, $J=2.2\text{Hz}$); mass spectrum (EI) 356 (M^+), 195, 162, 134, 122. Anal. Calcd. for $\text{C}_{16}\text{H}_8\text{N}_2\text{O}_8$: C, 53.94; H, 2.26; N, 7.86. Found: C, 52.18; H, 2.29; N, 7.51.

Ester Cleavage Assays.

Cleavage of DNP coumarin esters 14 and 15 by the thiol-containing antibodies was assayed in the

presence and absence of 0.1 μM NaCl, 50 mM sodium phosphate, pH 7.0, with 24 μM dithiothreitol at 10°C. The release of free coumarin was quantitated fluorometrically, exciting at 355 nm and measuring emission at 455 nm. The S-thiopyridyl Fab was first dialyzed against assay buffer, 30 min. prior to the assays, an aliquot of the thiopyridyl Fab (0.15 mg in 0.19 ml) was reduced with 3.8 mM dithiothreitol at 20°C. For each assay, reduced, thiolated Fab (15 μg in 18.8 μl) was diluted with assay buffer (2.95 ml) so that the net concentrations of thiolated Fab and of dithiothreitol were 0.1 μM and 24 μM , respectively. After equilibrating, the substrate was added (30 μl of a stock solution in acetonitrile) and the solution mixed for 10 sec. before monitoring fluorescence change. Antibody rates were corrected by subtracting the rate of cleavage in the absence of Fab.

The antibody affinity-labeled with 2 was found to accelerate the cleavage of ester 14 by a factor of 6×10^4 over the cleavage reaction in equimolar dithiothreitol. The reaction kinetics are consistent with the formation of a Michaelis complex (Fig. 6). The kinetic constants k_{cat} and K_m for the reaction are 0.87 min^{-1} and 1.2 μM , respectively. The thiolysis reaction was competitively inhibited



by DNP-glycine with a K_i of 8 μM . Neither the uncleaved affinity-labeled antibody nor the iodoacetamide-alkylated antibody accelerated the rate of the thiolysis reaction above the background rate. The stoichiometry of product release corresponded to 1.0 coumarin to 1.0 Fab-SH. Addition of hydroxylamine to the reaction buffer did not lead to multiple

turnovers. However, the introduction of both a thiol and a general base into the label may lead to a catalytic system (Bruice (1959) J. Am. Chem. Soc. 81:5444 and Street et al. (1985) J. Am. Chem. Soc. 107:7669). Interestingly, thiolated Fab labeled with bromoketone 4 did not hydrolyze esters 6, 7, or DNP-acetate. Fluorescence quenching experiments revealed that the derivatization Fab did not bind the substrate with appreciable affinity, presumably as a result of steric congestion of the antibody combining site (Fig. 7).

Derivatization with a Spectroscopic Probe.

In order to demonstrate that the thiolated Fab can be further derivatized with a reporter molecule, N-fluoresceinthioureido-2-mercaptoethylamine (Zackerman et al. (1987) Nucleic Acids Res. 15:5305) was selectively introduced via a disulfide exchange reaction (Fig. 3). The S-thiopyridyl Fab labeled with 2 (9.0 nanomoles in 0.50 ml of 0.1 M sodium phosphate, pH 7.3) was treated with N-fluoresceinthioureido-2-mercaptoethylamine (52 nanomoles in 0.50 of 0.1 M triethylammonium acetate, pH 7.5, containing 10% acetonitrile) at 23°C. The reaction was monitored by release of thiopyridone at 343 nm (Grasseti and Murray (1967) Arch. Biochem. Biophys. 119:41). After three hours, no additional thiopyridone was released and the mixture was dialyzed exhaustively against assay buffer (50 mM NaCl, 50 mM sodium phosphate, pH 7.0). The resulting adduct was isolated in 84% overall yield, with greater than 90% incorporation of the fluorophore.

Fluorescence Quenching Binding Assay.

Addition of the ligand DNP-glycine to the fluorescein-Fab adduct resulted in a decrease in fluorescence, providing a direct assay of ligand binding (Fig. 7). Fluorescence quenching experiments

were carried out at 10°C using 492 nm for excitation and measuring emission at 521 nm. The fluorescein-Fab adduct was diluted with assay buffer (50 mM NaCl, 50 mM sodium phosphate, pH 7.0) to 0.10 μ M. Aliquots of 2,4-DNP-glycine were added and, after mixing, the fluorescence observed. The association constant of DNP-glycine to the fluorescein-Fab adduct was determined to be $3.0 \times 10^5 \text{ M}^{-1}$, almost identical to the K_A of DNP-glycine to underivatized MOPC 315, $2.0 \times 10^5 \text{ M}^{-1}$. Addition of DNP-glycine to a solution of one equivalent free N-fluoresceinthioureido-2-mercaptoethylamine and underivatized Fab (each at 0.10 μ M) resulted in no detectable fluorescence change (Fig. 7).

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method for introducing an active
functionality to a polypeptide proximate a binding
5 site, said method comprising:
combining the polypeptide with a ligand
capable of binding to the binding site, said ligand
having a first reactive group cleavably attached
thereto;
10 covalently attaching the first reactive group
to an amino acid side chain on the polypeptide;
cleaving the first reactive group from the
ligand, whereby a moiety of the reactive group remains
bound to the polypeptide through the amino acid side
15 chain; and
removing the ligand from the polypeptide.
2. A method as in claim 1, wherein the
remaining moiety is the active functionality and is
20 selected from the group consisting of catalytic
functionalities and reactive functionalities.
3. A method as in claim 2, wherein the
moiety is a thiol.
25
4. A method as in claim 1, wherein cleavage
of the reactive group provides a moiety which is
selected from the group consisting of diazoketones,
aromatic azides, diazonium, thiol, α -bromoketone,
30 epoxide, and aldehyde.
5. A method as in claim 4, further
comprising covalently attaching a second functionality
to the reactive group moiety, wherein the second
35 functionality is the active functionality selected from
the group consisting of catalytic functionalities,

reactive functionalities, labeling groups, and
chemotherapeutic agents.

5 6. A method as in claim 2 or 5, wherein the
active functionality is a catalytic functionality
selected from the group consisting of acids, bases,
enzyme cofactors, metal complexes, photoactive
molecules, nucleophiles, electrophiles, and redox
active molecules.

10

 7. A method as in claim 2 or 5, wherein the
active functionality is a reactive functionality
selected from the group consisting of alkylating
agents, oxidizing agents, reducing agents, hydrolytic
15 agents, and photoactive agents.

 8. A method as in claim 5, wherein the
active functionality is a detectable label selected
from the group consisting of fluorophores,
20 chemiluminescers, bioluminescers, dyes, spin labels,
flavins, piezoelectric molecules and biotin.

 9. A method as in claim 5, wherein the
active functionality is a chemotherapeutic agent
25 selected from the group consisting of toxins, toxin
fragments, bactericides, radical scavengers, radical
generators, alkylating agents, neurotransmitters,
radionuclides, antiviral compounds, antifungal
compounds, antineoplastic agents, antimycoplasmal
30 agents, and heavy metals.

 10. A method as in claim 1, wherein the
polypeptide is selected from the group consisting of
antibodies, antibody fragments, enzymes, enzyme
35 fragments, hormones, lymphokines, lectins, avidin,
proteins of the MHC, T-cell receptors, G proteins,
neurotransmitter receptors, and DNA binding proteins.

11. A method for preparing a polypeptide having an active functionality proximate a binding site, said method comprising:

5 producing a polypeptide having a predetermined amino acid sequence which is substantially the same as at least a fragment of an exemplary polypeptide having a binding site, except that at least one amino acid has been added or substituted at a preselected location proximate the
10 binding site; and

covalently attaching an active functionality selected from the group consisting of reporter molecules and chemotherapeutic agents to a side chain of the added or substituted amino acid.
15

12. A method as in claim 11, wherein the added or substituted amino acid is selected from the group consisting of arginine, aspartate, cysteine, methionine, glutamate, histidine, lysine, serine, threonine, tryptophan, and tyrosine.
20

13. A method as in claim 11, wherein the added or substituted amino acid is other than a naturally-occurring amino acid.
25

14. A method as in claim 13, wherein the other than a naturally-occurring amino acid has a side chain selected from the group consisting of aryl amines, alkyl amines, aldehydes, carboxylates, and α - β unsaturated enones.
30

15. A method for modifying a polypeptide having a known amino acid sequence which defines a binding site, said method comprising:
35

expressing a DNA sequence encoding an amino acid sequence which is substantially the same as an amino acid sequence of the unmodified polypeptide

except that at least one amino acid has been added or substituted at a preselected location proximate the binding site;

5 recovering the expression product; and
covalently attaching an active functionality to a side chain of the added or substituted amino acid.

10 16. A method as in claim 15, wherein the added or substituted amino acid is selected from the group consisting of arginine, aspartate, cysteine, methionine, glutamate, histidine, lysine, serine, threonine, tryptophan, and tyrosine.

15 17. A method as in claim 12 or 16, the at least one amino acid is a cysteine having a thiol side chain and the active functionality is attached by disulfide exchange, alkylation, or electrophilic addition.

20 18. A method as in claim 12 or 16, wherein the amino acid is a histidine having an imidazole side chain and the active functionality is attached by reaction with an electrophilic reagent selected from the group consisting of α -haloketones, diazoketones,
25 and alkyl halides.

30 19. A method as in claim 12 or 16, wherein the amino acid is a lysine having a primary amine side chain and the active functionality is attached by reaction with an electrophilic reagent selected from the group consisting of α -haloketones, isothiocyanates, aryl sulfonyl halides, or by reaction with an aldehyde.

35 20. A method as in claim 12 or 16, wherein the amino acid is a tyrosine having a phenolic side chain and the functionality is attached by alkylation with an aryl diazonium compound or an α -haloketone, or

by nitration or reduction followed by acylation or alkylation.

5 21. A method as in claim 12 or 16, wherein the amino acid is aspartate or glutamate having carboxyl side chains and the functionality is attached by amino addition using an activating agent such as a carbodiimide or by alkylation with a diazo alkane.

10 22. A method as in claim 12 or 16, wherein the amino acid is serine or threonine having an aliphatic hydroxyl side chain and the functionality is attached by condensation with an α -halo ketone or an aryl sulfonylhalide.

15 23. A method as in claim 12 or 16, wherein the amino acid is tryptophan having indole side chain and the functionality is attached by oxidation of the tryptophane followed by alkylation or acylation.

20 24. A polypeptide having a binding site and at least one active functionality covalently attached to an amino acid side chain wherein said binding site is capable of forming a complex with a preselected
25 target ligand and said active functionality is a reporter molecule or a chemotherapeutic agent whereby the active functionality and the target ligand are able to interact without substantial loss of binding
30 affinity between the polypeptide and ligand.

 25. A polypeptide as in claim 24, wherein said polypeptide is an antibody or antibody fragment.

35 26. An antibody or antibody fragment as in claim 24, wherein the active functionality is a reporter molecule, wherein the detectability of the

reporter molecule is modulated by binding of the antibody to the target ligand.

5 27. An antibody or antibody fragment as in claim 26, wherein the detectable label is selected from the group consisting of fluorophores, chemiluminescers, bioluminescers, dyes, spin labels, flavins, piezoelectric molecules, and biotin.

10 28. An antibody or antibody fragment as in claim 24, wherein the active functionality is a chemotherapeutic agent, wherein activity and specificity of the chemotherapeutic agent against the target ligand is enhanced.

15 29. An antibody or antibody fragment as in claim 28, wherein the chemotherapeutic agent is selected from the group consisting of toxins, bactericides, radical scavengers, radical generators, 20 alkylating agents, neurotransmitters, radionuclides, antiviral compounds, antifungal compounds, antineoplastic agents, antimycoplasmal agents, and heavy metals.

25 30. A polypeptide having the formula:
P-X-Y-Z, wherein:

P is a polypeptide having a binding site and being selected from the group consisting of antibodies, antibody fragments, enzymes, enzyme fragments, 30 hormones, lymphokines, lectins, avidin, proteins of the MHC, T-cell receptors, G proteins, neurotransmitter receptors, and DNA binding proteins;

X is an amino acid side chain, said side chain being within about 20 Å of the binding site;

35 Y is a covalent linking group selected from the group consisting of disulfide, ester, amine, and diazo; and

Z is an active functionality selected from the group consisting of reporter molecules and chemotherapeutic agents, Z being attached to P through X and Y and being substantially absent from other locations on P.

31. A polypeptide as in claim 30, wherein the active functionality Z is a reporter molecule selected from the group consisting of fluorophores, chemiluminescers, bioluminescers, dyes, spin labels, flavins, piezoelectric molecules, and biotin.

32. A polypeptide as in claim 30, wherein the active functionality Z is a chemotherapeutic agent selected from the group consisting of toxins, bactericides, radical scavengers, radical generators, alkylating agents, neurotransmitters, radionuclides, antiviral compounds, antifungal compounds, antineoplastic agents, antimycoplasmal agents, and heavy metals.

33. A polypeptide as in claim 30, wherein P is selected from the group consisting of antibodies, antibody fragments, enzymes, enzyme fragments, hormones, lymphokines, lectins, avidin, proteins of the MHC, T-cell receptors, G proteins, neurotransmitter receptors, and DNA binding proteins.

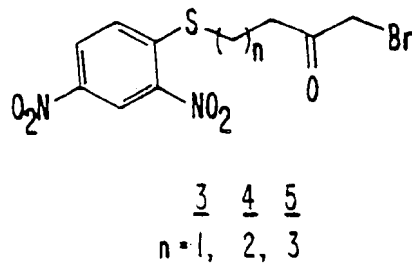
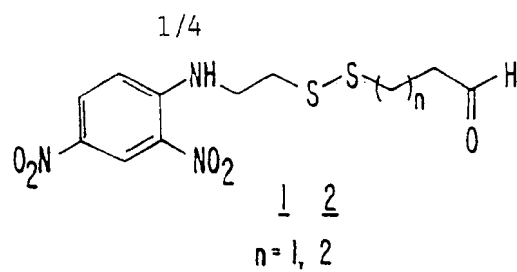


FIG. 1.

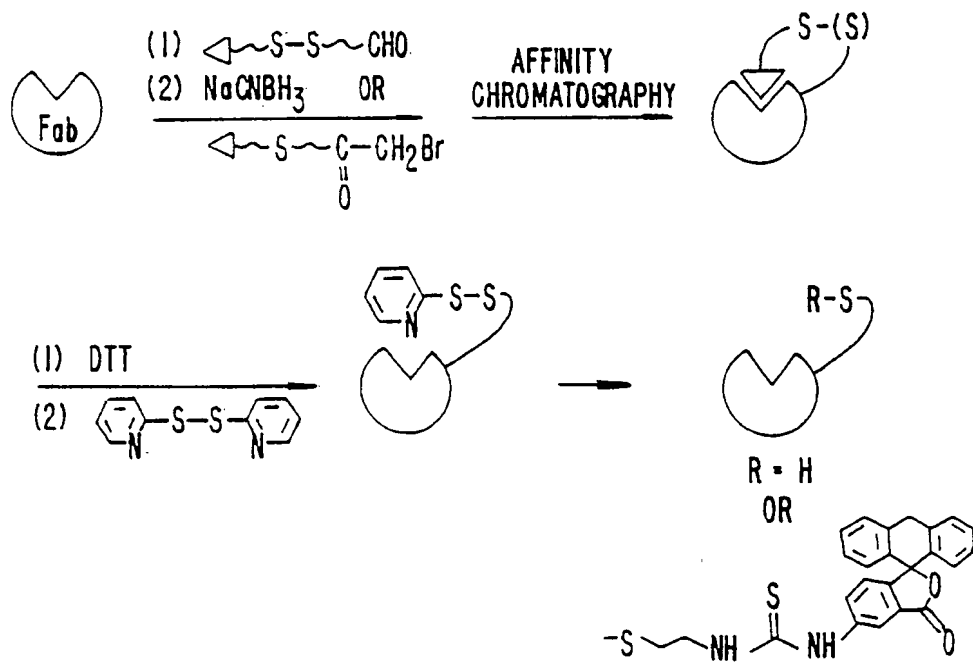


FIG. 3.

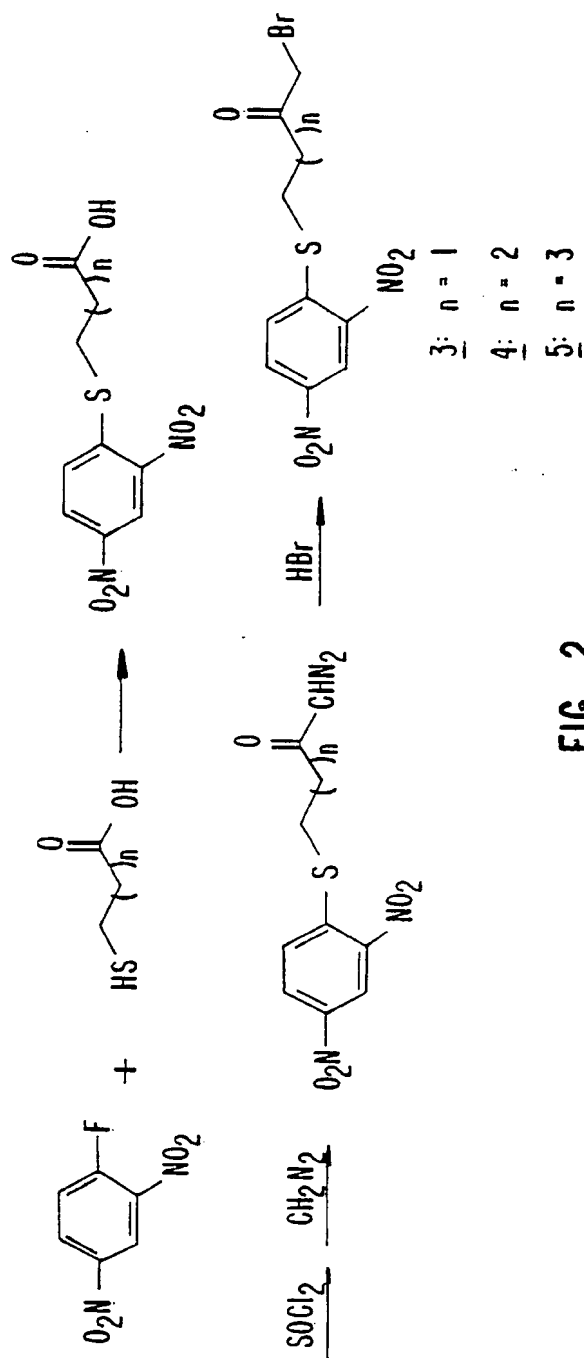
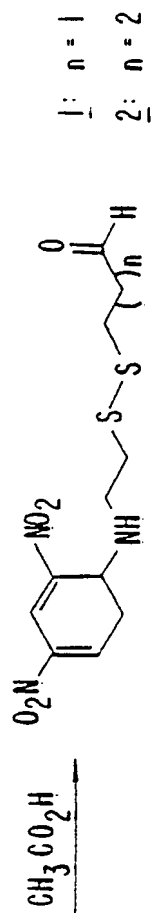
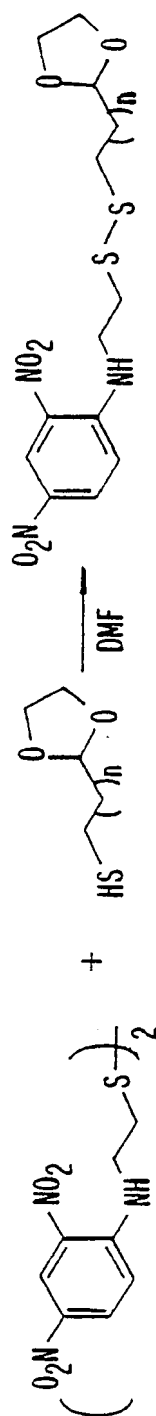


FIG. 2.

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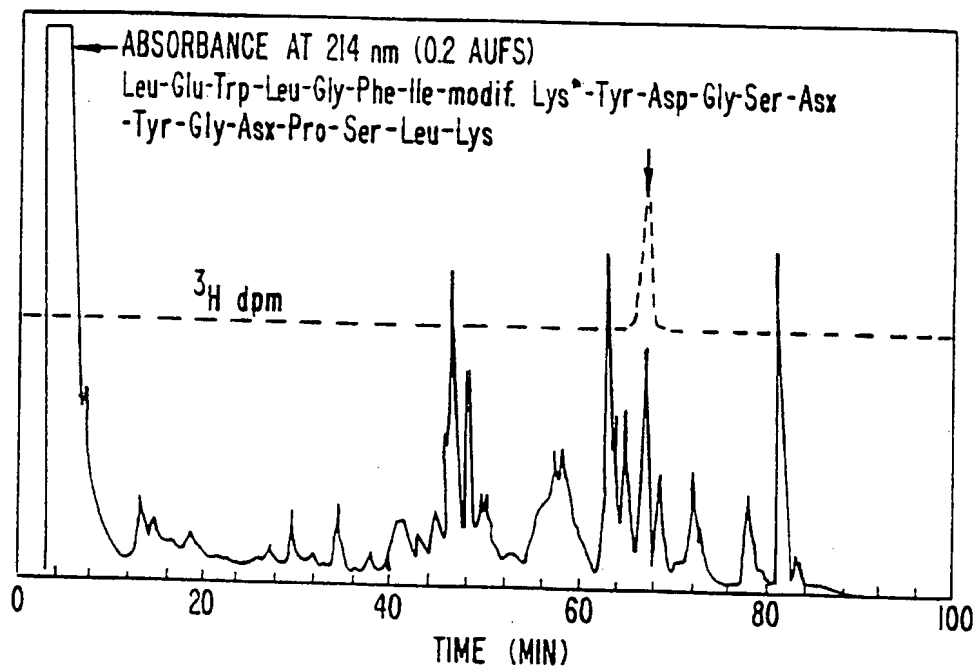


FIG._4.

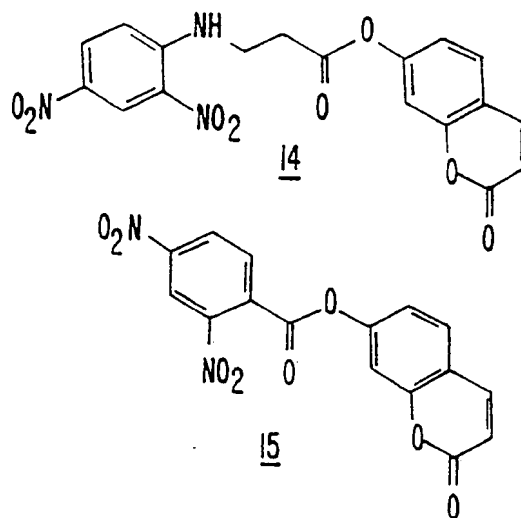


FIG._5.

SUBSTITUTE SHEET

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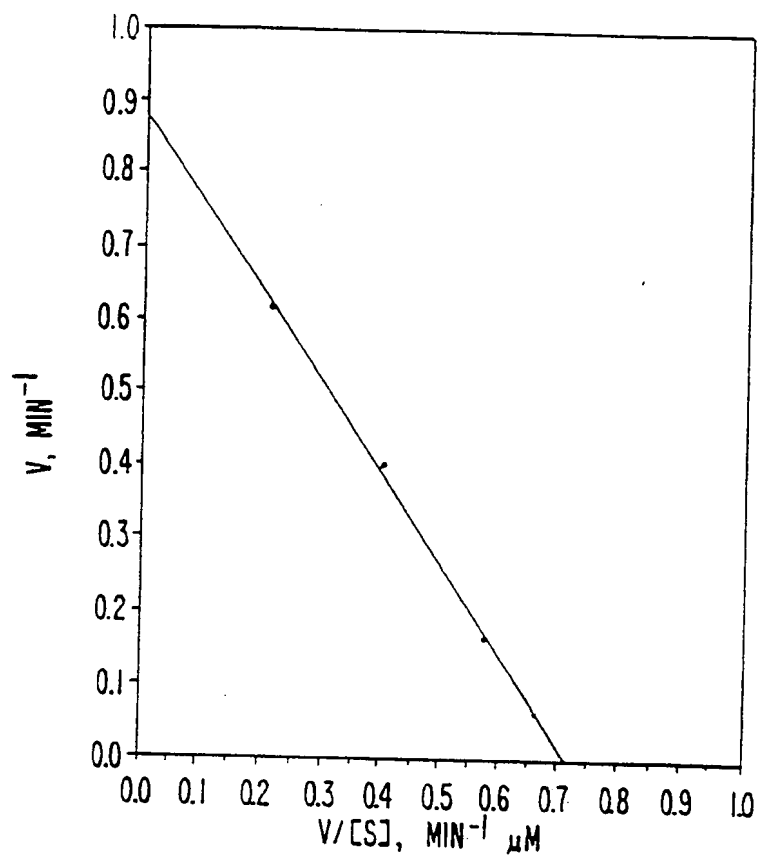


FIG. 6.

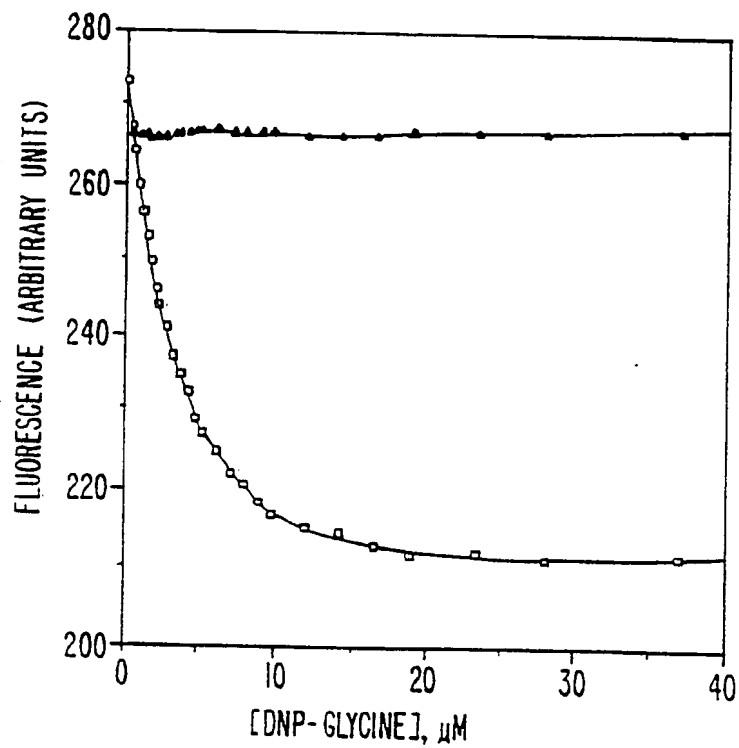


FIG. 7.

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/05257

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC IPC(5) C07K 17/06; C12N 9/96 U.S. CL. 530/390; 435/188						
II. FIELDS SEARCHED <div style="text-align: center; margin-top: 10px;">Minimum Documentation Searched ⁷</div> <table border="1" style="width: 100%; border-collapse: collapse; margin-top: 10px;"> <tr> <th style="width: 25%;">Classification System</th> <th style="width: 75%;">Classification Symbols</th> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">U.S.</td> <td style="padding: 5px;">530/389,408; 424/85.91; 436/537, 547</td> </tr> </table> <div style="text-align: center; margin-top: 10px;">Documentation Searched other than Minimum Documentation to the extent that such Documents are Included in the Fields Searched ⁸</div>			Classification System	Classification Symbols	U.S.	530/389,408; 424/85.91; 436/537, 547
Classification System	Classification Symbols					
U.S.	530/389,408; 424/85.91; 436/537, 547					
COMPUTER DATA BASES: APS, CAS, BIOSIS						
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹						
Category [*]	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³				
Y	US, A, 4,059,685 (JOHNSON) 22 November 1977 See column 8, lines 40-50.	1				
X	EP, A, 0187658 (HARBER) 16 July 1986, See pages 506, 12-13, 15 and 19-20.	1-6, 10				
X	US, A, 4,722,906 (GUIRE) 2 FEBRUARY 1988 See columns 2-6.	1-6,10				
Y	D.R. COREY, "Generation of a Hybrid Sequence-Specific Single-Stranded Deoxyribonuclease", <u>Science</u> volume 238, published 4 December 1987, see pages 1401-1402.	1-6,10				
Y	R.M. BAUM, "Catalytic Antibodies Open up New Strategy for Protein Engineering", <u>C & EN</u> , published 6 April 1987, see pages 31 and 33.	1				
P,X	I. AMATO, "Teaching Antibodies New Tricks," <u>Science News</u> volume 136, published 2 September 1989. See pages 153 and 155.	1-6,10				
(Continued on extra sheet #1)						
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>[*] Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance.</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"Δ" document member of the same patent family</p> </div> </div>						
IV. CERTIFICATION						
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report				
5 MARCH 1990		27 MAR 1990				
International Searching Authority		Signature of Authorized Officer [*]				
ISA/US		KAY K. KIM HO NGUYEN				

III. DOCUMENTS CONSIDERED TO BE RELEVANT

- Y E.T. KAISER, "Chemical mutation of enzyme active enzyme active site, "Science volume 226, published 2 November 1984. See pages 505, 506 and 509-511. 1-6, 10
- P,X S.J. Pollack, "Introduction of nucleophiles and spectroscopic probes into antibody combining sites," Sciece volume 242, published 18 November 1988. See pages 1038-1040. 1-6, 10
- P,X S.J. Pollack, "A semisynthetic catalytic antibody", 1-6, 10
J. Am. Chem. Soc. volume 111, published 1989.
See pages 1929 and 1930.

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

P,Y	B. S. GREEN, "Catalytic monoclonal antibodies tailor-made, enzyme-like catalysts for chemical reactions", <u>TIBTECH</u> VOLUME 7, published November 1989. See p. 308.	1-6, 10
Y	D. GIVOL, "Affinity labeling and cross-linking of the heavy and light chains of a myeloma protein with anti-2,4-dinitrophenyl activity", <u>Biochemistry</u> volume 10, number 18, published 1971. See pages 346, 3464 and 3466.	1-6, 10

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _____ because they relate to subject matter ¹² not required to be searched by this Authority, namely:

2. ☐ Claim numbers _____ because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out ¹³, specifically:

3. ☐ Claim numbers _____ because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²

This International Searching Authority found multiple inventions in this international application as follows:

SEE ATTACHMENT

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

1-6 and 10

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

Group I, claims 1-10 and 24-33 drawn to various protein conjugates and a method of preparing such, classified in classes 530, 424, and 435, subclasses 402, 85.91, and 188 respectively.

Group II, claims 11-14 and 17-23 drawn to a method of preparing modified protein conjugates, classified in class 530, subclass 345 and 402.

Group III, claims 15-23 drawn to a method of preparing modified protein conjugates by recombinant processes, classified in classes 530 and 435, subclasses 402 and 172.3 respectively.

Each of these groups encompass distinct protein represented by the following: 1) Antibodies; 2) Enzymes; 3) Hormones; 4) Lymphokines; 5) Lectins; 6) Avidin; and 7) all other protein.

Each of the groups encompass distinct functional agents as follows: 1) catalytic agents; 2) labels/reporter molecules; and 3) chemotherapeutic agents.

Each of the groups encompass distinct reactive groups as follow: 1) thiol and sulfur-containing; 2) aldehydes and ketones; 3) amines/amides; 4) azo/diazonium/azides; and 5) halides.

Groups II and III encompass synthetic or recombinant amino acid additions or substitutions for: 1) basic amino acids; 2) acidic amino acids; 3) aliphatic-hydroxyl containing amino acids; 4) sulfur-containing amino acids.

Detailed Reasons for Holding Lack of Unity of Invention:

PCT Rule 13.2 permits claims to "a" (one) product and "a" (one) method of making and "a" (one) method of using said product. The invention as set forth in Group I constitutes such a combination, but there are no method of use claims. However, the protein conjugates of Group I encompass several distinct conjugate species wherein various physically and functionally distinct classes of protein are conjugated, via various distinct functional/heterofunctional linkages to various classes of agents usable therapeutically or diagnostically (i. e. chemotherapeutic agents, labels/reporter molecules, or catalytic agents).

Furthermore, the methods of Groups II and III also represent distinct method of preparing modified protein conjugates. In addition, these two groups encompass amino acid substitutions and/or additions that represent distinct protein modifications relative to the nature of the side-chains of the amino acids.

VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

There are no provisions in PCT Rule 13.2 for claims covering multiple products and multiple methods of making such products. As the first appearing group, the protein conjugates and method of making such wherein the specific protein are antibodies; wherein the reactive moiety is thiol and/or sulfur-containing groups; and wherein the agent conjugated to the protein are the chemotherapeutic agents will be searched. Applicant is invited to pay for the search of some or all of the additional conjugates wherein six(6) additional protein groups have been listed above; two(2) other groups of agents to which the protein is conjugated have been specified; two(2) other methods of preparing the protein are set forth above, wherein either of these methods encompass four(4) kinds of modifications.